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Functions of the AP-1 transcription factor Fra-2 in epidermal development and disease

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Doctoral thesis submitted to the Universidad Autónoma de Madrid for the degree of Doctor of Philosophy by M.Sci. in Molecular Biomedicine,

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Summary

Summary

Altered epidermal differentiation and the production of inflammatory cytokines and chemokines by epidermal cells are hallmarks of numerous skin diseases affecting over 25% of the human population. Here I have identified Fra-2, an AP-1 transcription factor, as a key regulator of terminal epidermal differentiation and cytokine expression in keratinocytes. Mechanistically, Fra-2 binds and transcriptionally regulates gene promoters of epidermal differentiation genes, located within the Epidermal Differentiation Complex (EDC). EDC gene promoters are co-occupied by the transcriptional repressor Ezh2. Fra-2 remains transcriptionally inactive in non-differentiated keratinocytes, where it was found mono- and dimethylated on lysine 104, and interacted with Ezh2. Upon keratinocyte differentiation, Fra-2 is C-terminally phosphorylated on serine 320 and threonine 322 by ERK1/2, leading to transcriptional activation. Thus, the induction of epidermal differentiation by Fra-2 is controlled by a dual mechanism involving Ezh2-dependent methylation and activation by ERK1/2-dependent phosphorylation. During skin morphogenesis in mice, epithelial-restricted, ectopic expression of Fra-2 induced EDC gene expression. Moreover, in a papilloma-prone background, reduced tumor burden was observed due to precocious keratinocyte differentiation by Fra-2 expression. Importantly, loss of Fra-2 in suprabasal keratinocytes is sufficient to cause skin barrier defects due to reduced expression of differentiation genes.

Besides impairing keratinocyte differentiation, loss of epidermal Fra-2 (*Fra-2*$^{\Delta ep}$) results in inflammation characterized by epidermal hyperplasia, infiltration of inflammatory cells into the dermis and epidermis and high serum cytokine levels. Additionally, Fra-2-deficient keratinocytes display increased p65/NF-κB activity and the concurrent removal of epithelial p65 partially attenuates the skin and systemic phenotype of *Fra-2*$^{\Delta ep}$ mutants. These findings identify an important function of epidermal p65 in initiating inflammatory processes. Additionally, a cell autonomous but indirect regulation of Fra-2 on the expression of the cytokine TSLP by keratinocytes was uncovered.

Interestingly, transplanting *Fra-2*$^{\Delta ep}$ skin onto immune-compromised SCID mice gave rise to the formation of skin papillomas, suggesting a tumor protective role of the acute inflammatory response observed in *Fra-2*$^{\Delta ep}$ skin.

My data demonstrate a cell-autonomous function of Fra-2 in epithelial homeostasis by regulating keratinocyte differentiation, p65 activity and epithelial cytokine expression, which are causally involved in the development of inflammatory skin diseases and skin tumors.

Resumen

Resumen

Las alteraciones en la diferenciación epidérmica y la producción de citoquinas inflamatorias y quimiocinas por las células epidérmicas son señas de identidad de numerosas enfermedades de la piel que afectan a más del 25% de la población humana. Aquí he identificado Fra-2, un factor de transcripción AP-1, como un regulador clave de la diferenciación epidérmica y la expresión de citoquinas en los queratinocitos. Mecanísticamente, Fra-2 se une y regula transcripcionalmente promotores de genes de diferenciación epidérmica ubicados dentro del Complejo de Diferenciación Epidérmica (EDC del inglés *Epidermal Differentiation Complex*). Los promotores de los genes EDC son co-ocupados por el represor transcripcional Ezh2. Fra-2 permanece transcripcionalmente inactiva en los queratinocitos no diferenciados, donde se encontró mono y dimetilado en la lisina 104, e interactuando con Ezh2. Tras la diferenciación de los queratinocitos, Fra-2 es fosforilado en el extremo C-terminal, en la serina 320 y la treonina 322, por ERK1/2, lo cual conduce a su activación transcripcional. Por lo tanto, la inducción de la diferenciación epidérmica por Fra-2 se controla mediante un doble mecanismo de metilación dependiente de Ezh2 y la activación por fosforilación dependiente de ERK1/2. Durante la morfogénesis de la piel en ratones, la expresión ectópica de Fra-2, restringida a células epiteliales, indujo la expresión de los genes EDC. Además, en ratones susceptibles a papilomas se observó una reducción de los tumores debido a una diferenciación precoz de queratinocitos por la sobreexpresión de Fra-2. Es importante destacar que la pérdida de Fra-2 en los queratinocitos suprabasales es suficiente para causar defectos de barrera de la piel debido a la reducción de la expresión de genes de diferenciación.

Además de alterar la diferenciación de los queratinocitos, la pérdida de Fra-2 (*Fra-2*$^{\Delta ep}$) en la epidermis resulta en una inflamación que se caracteriza por hiperplasia epidérmica, infiltración de células inflamatorias en la dermis y la epidermis y niveles de citoquinas séricas elevadas. Además, los queratinocitos deficientes en Fra-2 muestran una mayor actividad de p65/NF-kB y la eliminación simultánea de p65 epitelial atenúa parcialmente el fenotipo de la piel y sistémico de los mutantes *Fra-2*$^{\Delta ep}$. Estos hallazgos identifican una función importante de p65 epidérmico en la iniciación de los procesos inflamatorios. Además, se pudo constatar una regulación autónoma pero indirecta de Fra-2 sobre la expresión de la citoquina TSLP por los queratinocitos.

Sorprendentemente, el trasplante de piel *Fra-2*$^{\Delta ep}$ en ratones inmunocomprometidos (SCID) dio lugar a la formación de papilomas en la piel, lo que sugiere un papel protector frente a tumores de la respuesta inflamatoria aguda observada en la piel *Fra-2*$^{\Delta ep}$.

Resumen

Mis datos demuestran una función autónoma de Fra-2 en la homeostasis epitelial mediante la regulación de la diferenciación de queratinocitos, la actividad de p65 y la expresión de citoquinas epiteliales que están implicadas causalmente en el desarrollo de enfermedades inflamatorias de la piel y tumores de piel.

Abbreviations

Abbreviations

AD	Atopic Dermatitis
AK	Actinic Keratosis
AP-1	Activator Protein 1
AP-2	Activating Protein 2
Arnt	Aryl hydrocarbon receptor nuclear translocator
ATF	Activating Transcription Factor
BCC	Basal cell carcinoma
Bcl-2	B-cell lymphoma 2
BM	basement membrane
bp	base pair
bZIP	basic leucine zipper
C/EBP	CCAAT/enhancer binding protein
Ca2+	calcium
CBP	CREB-Binding Protein
CD4	cluster of differentiation 4
CD45	cluster of differentiation 45
CD8	cluster of differentiation 8
CE	cornified envelope
ChIP	chromatin immunoprecipitation
coIP	co-immunoprecipitation
DC	dendritic cell
DETC	dendritic epidermal T cell
DKO	double knock-out
DMBA	7,12-Dimethylbenz[a]anthracene
DNA	deoxyribonucleic acid
E17.5	embryonic day 17.5
ECM	extracellular matrix
EDC	Epidermal Differentiation Complex
ELISA	Enzyme Linked Immunosorbent Assay
EGFR	Epidermal Growth Factor Receptor
ERK	Extracellular-Signal-Regulated Kinase
FBS	fetal bovine serum
FGF	fibroblast growth factor
Flg	Filaggrin

Abbreviations

Fra-2/p65$^{\Delta ep}$	knock-out of Fra-2 and p65 in all epidermal cells
Fra-2$^{\Delta ep}$	knock-out of Fra-2 in all epidermal cells
Fra-2$^{\Delta sb}$	knock-out of Fra-2 in suprabasal cells
Fra-2$^{Ep\text{-}tetOFF}$	ectopic expression of Fra-2 in epithelial cells
Fra-2 K104F	Fra-2 mutant with substitution of lysine 104 to phenylalanine
Fra-2 S320A/T322A	Fra-2 mutant with substitution of serine 320 and threonine 322 to alanine
GEMM	genetically engineered mouse model
GOF	gain of function
Grhl3	grainyhead-like 3
H&E	hematoxylin and eosin
H2	histone 2
H3	histone 3
H3K27me3	histone 3 lysine 27 trimethylation
H3K4me3	histone 3 lysine 4 trimethylation
HF	hair follicle
HIF1α	hypoxia-inducible factor 1 alpha
IF	immunofluorescence
IgE	Immunglobulin E
IHC	immunohistochemistry
IL-13	interleukin 13
IL-17	interleukin 17
IL-1ß	interleukin 1 beta
IL-1α	interleukin 1 alpha
IL-4	interleukin 4
IL-6	interleukin 6
IP	immunoprecipitation
IκB	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor
JNK	c-Jun N-terminal kinase
K14-HPV	transgenic mice that expresses human papillomavirus in keratin 14 expressing tissues
K5-SOS-F	transgenic mice that express a dominant form of Son of Sevenless (SOS-F) in keratin 5 expressing tissues

Abbreviations

KGF	keratinocyte growth factor
KLF4	Kruppel-like factor 4
LB	lamellar bodies
LCE	late cornified envelope gene
Lcn2	Lipocalin-2
LIF	leukemia inhibitory factor
LOF	loss of function
LOH	loss of heterozygosity
Lor	Loricrin
MAF	musculoaponeurotic fibrosarcoma
MAP kinase	mitogen-activated protein kinase
MS	Mass spectrometry
mKC	mouse keratinocyte
mRNA	messenger ribonucleic acid
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NOD scid gamma	immunodeficient mice that lack mature T cells, B cells, and natural killer cells
OCT	Optimal Cutting Temperature
o/n	over night
P2	postnatal day 2
P6	postnatal day 6
PcG	Polycomb Group
Pen/Strep	Penicillin/Streptomycin
PFA	paraformaldehyde
PKC	protein kinase C
PRC	Polycomb Repressive Complex
PTCH	Patched
PTM	posttranslational modification
RHD	Rel homology domain
RT	room temperature
S100A8	S100 calcium binding protein A8
S100A9	S100 calcium binding protein A9
SCC	squamous cell carcinoma
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis

Abbreviations

SHH	sonic hedgehog
SMO	smoothened
STAT3	signal transducer and activator of transcription 3
TACE	TNF alpha converting enzyme
Tchhl1	trichohyalin-like 1
TEWL	transepidermal water loss
TF	transcription factor
Th cell	T helper cell
Timp3	tissue inhibitor of metalloproteinase 3
TLR	Toll-like receptor
TNF	tumor necrosis factor
TPA	12-O-Tetradecanoylphorbol-13-acetate
TRE	TPA responsive element
TrxG	Trithorax Group
TSLP	Thymic Stromal Lymphopoietin
WB	Western blot

Introduction

1. TRANSCRIPTIONAL MECHANISMS

One fundamental goal of biological sciences is to understand how different cell fates are acquired during development and how cellular identity is maintained in adulthood. The set of genes that are transcribed largely defines the cell. Mammals contain hundreds of cell types, and most of these have yet to be studied with respect to the set of transcripts they contain. The gene expression programs that regulate somatic tissue development are controlled by coordinated interactions of tissue specific transcription factors, cofactors, and chromatin regulators **(Figure 1-I)** (Young, 2011).

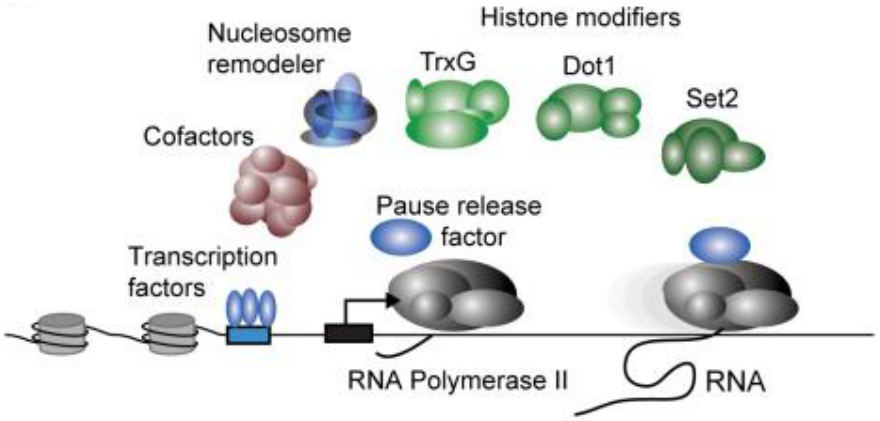


Figure 1-I. General transcriptional machinary at active gene promoters. *Adopted from Young, 2011.*

1.1. Transcription factors

In mammals, transcription factors (TF) make up the largest single class of proteins encoded in the genome, representing approximately 10% of all protein coding genes (Levine and Tjian, 2003). TFs integrate extracellular signals and function as gatekeepers between the cell surface and the nucleus. They recognize specific DNA sequences and either promote or prevent the recruitment of RNA polymerase II, the enzyme converting genetic information from DNA to messenger RNA (mRNA), a process termed transcription (Jacob and Monod, 1961). Many of these transcriptional regulators cluster into large families defined by highly homologous DNA-binding domains that have the capacity to bind highly similar DNA sequences. A small number of key TFs dominate the control of gene expression programs. They function in a temporally and spatially appropriate pattern at correct levels for normal tissue development (Cole and Young, 2008).

The activities of TFs themselves are often regulated through one or more posttranslational modifications (PTMs). PTMs constitute covalent chemical changes - such as phosphorylation, methylation, acetylation, sumoylation or glycosylation - to specific

residues in the protein sequence. These PTMs orchestrate every activity of a TF from subcellular localization to protein-protein interactions, sequence-specific DNA binding, transcriptional regulatory activity, and protein stability (Filtz et al., 2014).

1.1.1. Transcription factor phosphorylation

To activate or repress gene expression, TFs must be located in the nucleus, bind DNA, and interact with the basal transcription apparatus. Most commonly, this is regulated by reversible phosphorylation. Phosphorylation is the addition of a phosphate group to a serine, threonine or tyrosine residue of a protein (Gonzalez et al., 1989; Hunter and Karin, 1992). Phosphorylation of a TF by several different kinases is a simple mechanism that allows different signals to converge at the same factor. For numerous TFs, phosphorylation increases protein stability, fosters interaction with transcriptional cofactors, promotes nuclear translocation and thereby increases its transcriptional activity (Gonzalez et al., 1989; Hill and Treisman, 1995; Treisman, 1994). However, there are other examples where phosphorylation decreases stability, inhibits the nuclear translocation or the DNA binding affinity of a TF (Cowley and Graves, 2000; Okamura et al., 2000). Phosphorylation of TFs is a regulatory mechanism that is both rapid and readily reversible. Furthermore, as a TF can be targeted by many protein kinases and phosphatases, phosphorylation can effectively integrate information carried by multiple signal transduction pathways, thus providing opportunities for further versatility and flexibility in gene regulation (Calkhoven and Ab, 1996; Chinenov and Kerppola, 2001; Karin and Hunter, 1995).

1.1.2. Transcription factor methylation

Methylation is the addition of a methyl group to a lysine or arginine residue of a protein. Once bound to DNA, p53, NFκB, STAT3, GATA4 and several other TFs are reversibly methylated on lysine residues by enzymes that also modify histones. These modifications profoundly affect the activity of TFs at these promoters, altering their stability, transactivation potency, and affinity for DNA, and thus affecting the strength and duration of gene expression. For example, methylation of GATA4 attenuates its transcriptional activity by reducing its interaction with a transcriptional cofactor (Chuikov et al., 2004; He et al., 2012; Kim et al., 2013; Levy et al., 2011).

1.1.3. AP-1 transcription factors

The AP-1 (activator protein 1) TF is a dimeric complex that comprises members of the Jun (c-Jun, JunB and JunD), Fos (c-Fos, FosB, Fra-1 and Fra-2), ATF (activating transcription factor) and MAF (musculoaponeurotic fibrosarcoma) protein families. The AP-1 complex can therefore form many different combinations of hetero- and homo-dimers, and this combination determines the promoter binding affinity and the genes that are regulated by AP-1 (Chinenov and Kerppola, 2001; Vogt, 2002). AP-1 proteins are termed basic leucine-zipper (bZIP) proteins because they dimerize through a leucine-zipper motif and contain a basic domain for DNA binding. AP-1 TFs recognize the consensus sequence TGAC/GTCA also termed the TPA-responsive element (TRE), because it is strongly induced by the tumor promoter 12-*O*-tetra-decanoylphorbol-13-acetate (TPA) **(Figure 2A-I)** (Angel and Karin, 1991).

The regulation of AP-1 activity is complex and occurs at different levels, including dimer composition, transcriptional and post-translational events, and interactions with co-activators or -repressors (Bakiri et al., 2002; Chang and Karin, 2001; van Dam and Castellazzi, 2001). The DNA binding of the AP-1 complex to the TRE sequence is rapidly induced by growth factors, cytokines and oncogenes (Angel and Karin, 1991).

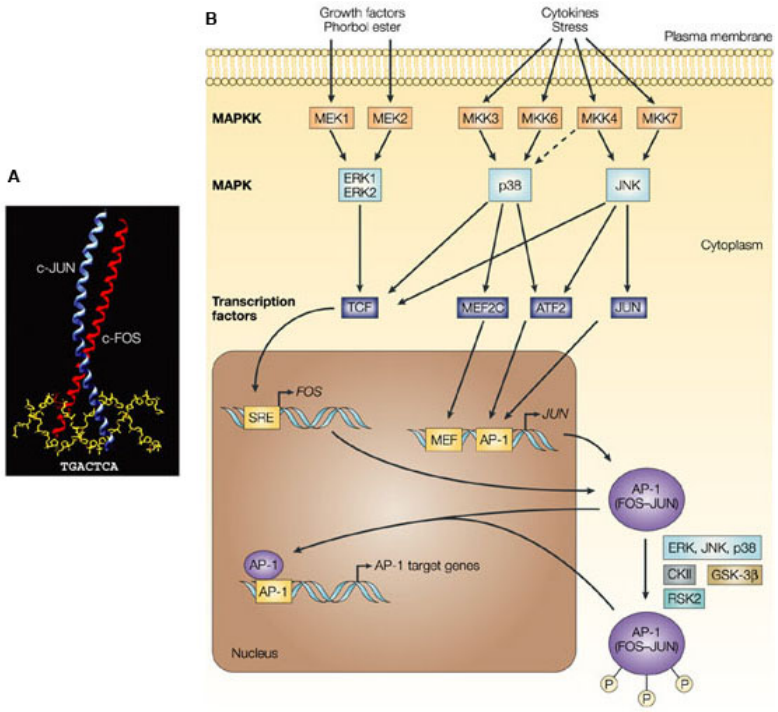


Figure 2-I. AP-1 transcription factor. A. Schematic representation of an AP-dimer bound to a TRE DNA element. B. Transcriptional and post-translational modifications of AP-1. *Adapted from Eferl and Wagner, 2003.*

AP-1 proteins are phosphorylated and activated by mitogen activated protein (MAP) kinases including Jun N-terminal kinase (JNK), p38 and Extracellular Signal-Regulated Kinase (ERK) (Eferl and Wagner, 2003). ERKs phosphorylate c-Fos, Fra-1 and Fra-2, thereby enhancing their DNA binding activity, as they heterodimerize with Jun proteins (Chang and Karin, 2001). JNKs phosphorylate c-Jun and less efficiently JunD, but they do not phosphorylate JunB **(Figure 2B-I)** (Kallunki et al., 1996).

AP-1 TFs control distinct biological processes including cellular proliferation, differentiation, oncogenic transformation, apoptosis and inflammation (Eferl and Wagner, 2003; Zenz et al., 2008).

1.1.4. NF-κB transcription factors

In mammals, the Nuclear Factor kappa-light-chain-enhancer of activated B cells (NF-κB) TF family consists of five proteins, p65 (RelA), RelB, c-Rel, p105/p50 (NF-κB1), and p100/p52 (NF-κB2) that associate with each other to form distinct transcriptionally active homo- and heterodimeric complexes. They all share a conserved 300 amino acid N-terminal Rel homology domain (RHD). Sequences within the RHD are required for dimerization, DNA binding, interaction with inhibitor of kappa B (IκB), as well as nuclear translocation (Baldwin, 1996).

NF-κB signaling plays critical roles in inflammation, immunity, cell proliferation, differentiation, and survival (Barnes and Karin, 1997; DiDonato et al., 2012). Inducible NF-κB activation depends on phosphorylation-induced proteosomal degradation of IκB proteins, which retain inactive NF-κB dimers in the cytosol in unstimulated cells. The majority of the diverse signaling pathways that lead to NF-κB activation converge on the IκB kinase (IKK) complex, which is responsible for IκB phosphorylation and is essential for signal transduction to NF-κB. Additional regulation of NF-κB activity is achieved through various PTMs of the core components, such as phosphorylation or acetylation of p65, which increase its transcriptional activity (Ghosh and Karin, 2002).

Over the past years, inflammation has emerged as a hallmark of tumor growth and progression. Of various signaling cascades involved in inflammatory processes, the NF-κB pathway has received extensive attention as a key mediator providing a link between inflammation and tumorigenesis (DiDonato et al., 2012; Karin, 2006).

1.2. Transcriptional cofactors

TFs typically bind cofactors, which are protein complexes that contribute to activation (co-activators) and repression (co-repressors) but do not have DNA binding properties of their own (Roeder, 1998).

Most TFs are thought to contribute to transcription initiation by recruiting co-activators such as mediator, p300 or its homolog CREB-binding protein (CBP), which in turn bind and control the activity of the transcription initiation apparatus (Conaway et al., 2005; Malik and Roeder, 2005). p300/CBP is one of the most potent and versatile acetyltransferases. p300/CBP acetylates and regulates the activity of various transcription-related proteins besides histones, by transferring an acetyl group from acetyl-coenzyme A to the ε-amino group of specific lysine side chains, it regulates the activity of a multitude of proteins (Brownell and Allis, 1996; Gu and Roeder, 1997; Janknecht and Hunter, 1996).

1.3. Chromatin regulators

Eukaryotic genomes are packaged into nucleosomes, which provide a means to compact the genome and to influence gene expression (Kornberg and Thomas, 1974; Olins and Olins, 1974). Early and recent studies revealed that gene expression can be influenced by histone-modifying enzymes that acetylate, methylate, ubiquitinate and otherwise chemically modify nucleosomes. These modifications provide interaction surfaces for protein complexes that contribute to transcriptional control. Enzymes that remove these modifications are also typically present at active genes, producing a highly dynamic process of chromatin modification as RNA polymerase is recruited (Bannister and Kouzarides; Brownell et al., 1996; Campos and Reinberg, 2009; Gardner et al., 2011). Chromatin regulators are generally recruited to genes by DNA binding transcription factors, the transcription apparatus, or specific RNA species (Guenther and Young, 2010; Roeder, 2005; Surface et al., 2010).

1.3.1. Histone-modifiying enzymes

The chromatin regulators with the most important impact on somatic tissue development are histone-modifying enzymes, a group of epigenetic regulators required for the earliest stages of embryonic lineage commitment (Sauvageau and Sauvageau, 2010). These include the Polycomb group (PcG) protein complexes, which are antagonized by the Trithorax group (TrxG) proteins. The corresponding genes were first identified in Drosophila melanogaster as repressors and activators of Hox genes (Bracken and Helin, 2009; Ringrose and Paro, 2004; Schuettengruber et al., 2007). TrxG proteins catalyze

trimetylation of histone H3 on lysine 4 (H3K4me3) at the promoters of active genes and facilitate maintenance of active gene states during development (Jenuwein and Allis, 2001). PcG proteins associate to form two major complexes, which are Polycomb Repressive Complex (PRC) 1 and 2. The mammalian PRC1 complex consists of four main subunits: one of the Cbx proteins (Cbx2-4-6-7-8), Ring1 (Ring1A/B), PHC (PHC1-3) and PCGF (PCGF1-6) (Levine et al., 2002). On the other hand, the PRC2 complex is formed by three core subunits Ezh1/Ezh2, Eed and Suz12 (Cao et al., 2002). The catalytic function of PRC2 requires methyltransferase activities of either Ezh1 or Ezh2, with Ezh2 being more catalytically active (Margueron et al., 2008).

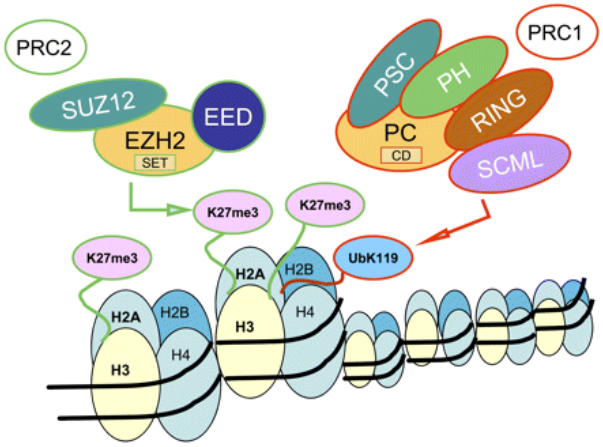


Figure 3-I. Polycomb-mediated gene silencing. *Adopted from Choi and Friso, 2010.*

PRC2 is recruited to chromatin and Ezh1/2 mediate the trimethylation of histone H3 on lysine 27 (H3K27me3). This histone mark presents a docking site for PRC1, which actively participates in gene silencing by monoubiquitination of H2A at lysine 119 and subsequent chromatin compaction **(Figure 3-I)** (Francis et al., 2004; Min et al., 2003; Simon and Kingston, 2009). H3K27me3 is found at genes that are silent but poised for activation at some later stage of development and differentiation (Orkin and Hochedlinger, 2011; Young, 2011).

Whether H3K27 methylation is the only way by which Ezh2/PRC2 controls transcription remains unclear. Since Ezh2 has other substrates beyond histone H3, it was proposed that Ezh2 might be involved in regulating tissue homeostasis by controlling the activity of these non-histone substrates, such as some TFs (He et al., 2012; Kim et al., 2013; Lee et al., 2012; Xu et al., 2012).

2. THE SKIN

The skin is emerging as an ideal model system to characterize the functional roles of transcriptional mechanisms that orchestrate organogenesis and adult tissue homeostasis. Due to its ability to regenerate throughout adulthood and the availability of excellent genetic tools, *in vivo* systems using mouse models offer a number of advantages to study gene functions during skin development and disease.

The skin is the body's largest organ in surface and weight. Mammalian skin comprises a stratified epithelium - the epidermis, a multilayered sheet of keratinocytes - and an underlying connective tissue, the dermis **(Figure 4-I)** (Frye and Benitah, 2012). Interactions between dermal cells and keratinocytes play a crucial role in the regulation of tissue morphogenesis, homeostasis and repair (Beck and Blanpain, 2012). The skin primarily serves as a protective barrier and prevents dehydration, mechanical trauma, microbial insults, and it is essential for temperature regulation, secretion and sensation. Complex interactions of different cell types found within the skin, orchestrated by coordinated transcriptional mechanisms, ensure these vital functions (Fuchs, 2009; Fuchs and Horsley, 2008; Watt, 2014).

2.1. The Dermis

The dermis is the fibrous connective tissue between the epidermis and the subcutaneous fat. Mesenchymal fibroblasts are the major cell type in the dermis and deposit the collagen and elastic fibres of the extracellular matrix (ECM) (Driskell et al., 2013; McLafferty et al., 2012). The dermis also contains adipocytes, blood and lymph vessels, nerve endings, hair follicles and glands. Immune cells, such as macrophages, mast cells, B- and T-lymphocytes and dendritic cells also populate the dermis (Gebhardt et al., 2011). Specialized resident immune cells found mainly in the dermis, including dendritic cells and γδ T cells, protect against invading pathogens (Watt and Fujiwara, 2011) **(Figure 4-I)**.

2.2. The Epidermis

Due to its constant exposure to the outer surface of the body, skin epithelia must rejuvenate constantly to maintain its barrier function, ensure long-term homeostasis and regenerate damaged areas. To fulfill these essential functions, the epidermis generates an elaborate array of supportive appendages, including hair follicles, sebaceous glands, sweat glands, and nails. Since terminally differentiated cells are continuously shed from the body surface, new cells have to replace the ones that are lost. In addition, hair follicles undergo re-occurring cycles of growth and regression. Epidermal homeostasis, hair

regeneration, and wound repair are maintained by several reservoirs of self-renewing stem cells located at distinct anatomical locations within the skin, that upon specific stimuli become activated, multiply and differentiate according to the needs of their particular compartment. The balance between proliferation and differentiation of keratinocytes is tightly regulated and the disruption of this balance causes several pathological conditions including inflammation and tumorigenesis (Blanpain and Fuchs, 2009, 2014; Fuchs, 2009; Hsu and Fuchs, 2012; Hsu et al., 2014).

Besides keratinocytes, three other cell types reside within the epidermis: Melanocytes, Langerhans cells and Merkel cells. Melanocytes are located in the lower part of the epidermis and synthesize melanin, the pigment that gives skin its natural color (Haass and Herlyn, 2005). Langerhans cells are dendritic cells (antigen-presenting immune cell) of the epidermis, also called dendritic epidermal T cells (DETC). They are present in all layers of the epidermis, but are most prominent in the spinous layer, where they take up and process microbial antigens to become fully functional (Romani et al., 2012) **(Figure 4-I)**. Merkel cells are oval receptor cells that have synaptic contacts with somatosensory neurons. These cells are involved in the sensation of light touch (Boulais et al., 2007).

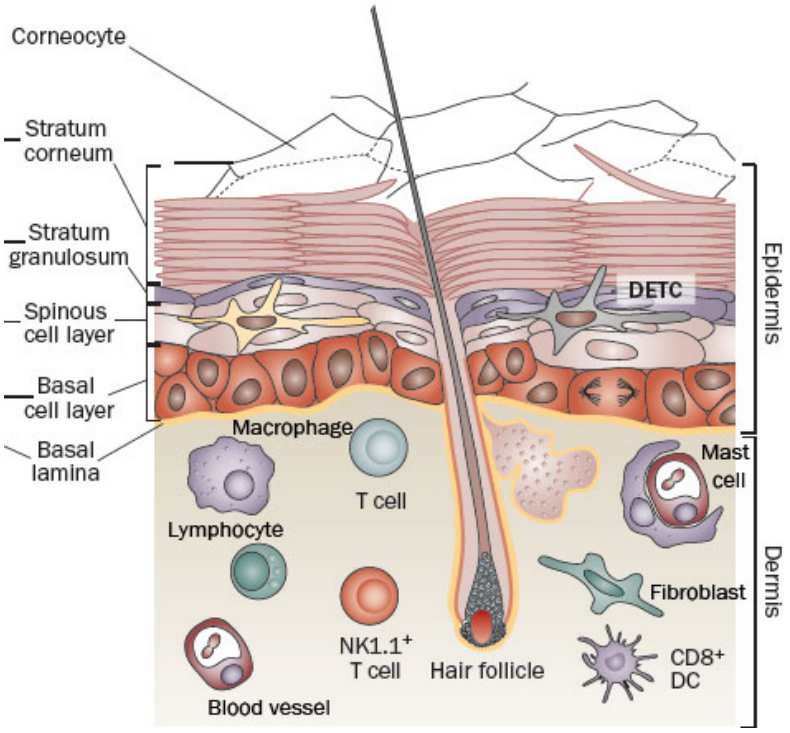


Figure 4-I. Compartments/cell types of the skin. *Adapted from Wagner et al., 2010.* Abbreviations: DC, dendritic cell; DETC, dendritic epidermal T cell; NK, natural killer cell.

2.2.1. Epidermal morphogenesis

In mice the program of epidermal morphogenesis begins at approximately embryonic day (E) 9.5 with a single layer of ectodermal cells that will undergo epidermal stratification at E14.5, resulting in the formation of a functional epidermal barrier at E18.5 (Blanpain and Fuchs, 2009; Koster and Roop, 2007).

Keratinocytes found within the innermost (basal) layer of this stratified epithelium rest upon a basement membrane (BM), which is rich in extra-cellular matrix proteins and growth factors and separates the epidermis from the underlying dermis. Basal cells, which express high levels of keratin 5 (K5), K14 and basal integrins, retain a population of progenitors that retain their proliferative potential and rely upon BM and mesenchymal stimuli to remain proliferative. Upon detachment from the BM, epidermal keratinocytes progress through several stages of differentiation that form three consecutive suprabasal layers: the spinous layer (*stratum spinosum*), the granular layer (*stratum granulosum*) and the outermost cornified layer (*stratum corneum)* **(Figure I4)** (Blanpain and Fuchs, 2006; Blanpain et al., 2007).

2.2.1.1. Basal-to-spinous transition

Each stage of differentiation is characterized by morphological and complex biochemical changes corresponding to the expression of different structural proteins. The major structural change at the basal-to-spinous transition, as proliferating basal keratinocytes become suprabasally mitotically inactive, is the switch from K5/K14 intermediate filaments in the basal layer to K1/K10 in the suprabasal layer. Spinous keratinocytes are attached together through desmosomes, which transmit physical forces onto K1 and K10 and build a scaffold for upper epidermal layers (Fuchs and Green, 1980; Koster and Roop, 2007).

2.2.1.2. Terminal differentiation

Upon subsequent migration, cells enter the granular layer, where the most abundant cornified envelope protein Loricrin (Lor) is expressed (Blanpain and Fuchs, 2009). Profilaggrin is also expressed at this stage of differentiation, and soon afterwards, it is proteolytically processed to generate Filaggrin (Flg), a protein that bundles keratin filaments into indestructible cables (Aho et al., 2012). Other terminal epidermal differentiation proteins are Trichohyalin-Like 1 (Tchhl1), and the Late Cornified Envelope proteins (LCEs). They are deposited on keratin intermediate filaments and increase mechanical strength (Fuchs and Horsley, 2008).

A multitude of genes necessary for terminal epidermal differentiation and cornification are found within the Epidermal Differentiation Complex (EDC), a gene-rich region of human chromosome 1q21, which corresponds to mouse chromosome 3. Interestingly, besides structural proteins required for skin barrier formation, this locus encodes chemoattractants such as S100 proteins, which are secreted by stressed keratinocytes and act on a multitude of immune cells (de Guzman Strong et al., 2010; Fessing et al., 2011; Segre, 2006).

2.2.1.3. Formation of the cornified envelope (CE) and skin barrier

Acquired just before birth and at the last stage of epidermal differentiation, the skin's barrier creates a surface seal essential for protecting animals against microbial infections and dehydration. As granular cells transit to the *stratum corneum*, their metabolic activity ceases, and an influx of calcium (Ca^{2+}) results in an activation of transglutaminases, which crosslink EDC gene products to generate the cornified envelope (CE) (Eckert et al., 2005). Concomitantly, lipids (free fatty acids, ceramides, cholesterol) first accumulate in lamellar bodies (LB), which are organelles that develop from the Golgi complex. LBs later fuse with the cell membrane to secrete their material into the extracellular environment. Secreted ceramides are covalently bound to the outside of the cornified envelope and assemble on this scaffold as multilayered lipid lamellae, thereby waterproofing the skin surface (Ishida-Yamamoto et al., 2004; Kalinin et al., 2001). Eventually, keratinocytes in the *stratum corneum* undergo apoptosis and are released from the surface of the epidermis (Blanpain and Fuchs, 2009). The program of epidermal differentiation is maintained throughout the lifespan of an organism and is regulated at several levels by numerous signaling pathways and transcription factors, as well as epigenetically. The functionality of the skin barrier in mice can be assessed by dye penetration assays – evaluation of the outside-in barrier - and by measuring the transepidermal water loss (TEWL), to measure the functionality of the inside-out barrier (Blanpain and Fuchs, 2009; Botchkarev et al., 2012; Eckert et al., 2011; Frye and Benitah, 2012; Truong and Khavari, 2007; Zhang et al., 2012).

2.2.2. Ca^{2+} induced *in vitro* epidermal differentiation

Mammalian epidermis displays a characteristic Ca^{2+} gradient, with highest levels in the granular and lowest levels in the basal layer, which is important for both permeability barrier homeostasis and epidermal differentiation (Menon et al., 1985). Much has been learned about Ca^{2+}-regulated differentiation from *in vitro* studies. Keratinocytes in low Ca^{2+} concentrations (0.05mM) proliferate but fail to differentiate. When switched to Ca^{2+}

concentrations above 0.1mM, the differentiation process is initiated. Keratinocytes rapidly undergo morphologic changes with the development of cell–cell contacts that are critical for the differentiation process **(Figure 5-I)**. The sustained increase in intracellular Ca^{2+} results in the activation of PKC (Protein kinase C) and upon activation of down-stream kinases and TFs, the sequential expression of K1 and K10, Lor and Flg. Therefore, "Ca^{2+} switch" experiments with undifferentiated, proliferating keratinocytes can be used to mimic epidermal differentiation *in vitro* to study transcriptional mechanisms (Bikle et al., 2013; Yuspa et al., 1989).

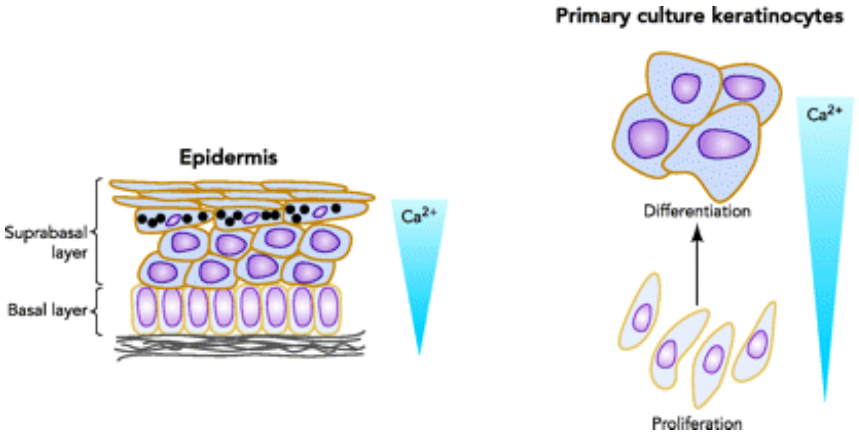


Figure 5-I. Ca^{2+} gradient *in vivo* and *in vitro*. *Adopted from Nakamura and Fukami, 2009.*

2.2.3. Transcriptional regulators in epidermal differentiation

2.2.3.1. TF functions in the basal-to-suprabasal transition

At the transcriptional level, the basal-to-spinous switch is controlled by p63 and the canonical Notch signaling pathway. The down-regulation of the epidermal master regulator p63, which is expressed in basal epidermal cells, presents a central event for the basal-to-spinous transition to happen (Dotto, 2009; Koster et al., 2004). The canonical Notch pathway is also crucial during the early steps of basal cell commitment to spinous cells. Ligands of Notch reside in the basal layer, and receptors for Notch are expressed suprabasally by spinous cells. Exceeding Notch activity promotes the fate of K1/K10-expressing spinous cells, while insufficient Notch signaling results in a diminution of this differentiated state (Blanpain et al., 2006; Rangarajan et al., 2001). Among Notch's target genes are the transcriptional repressor Hes1 and C/EBPs, which transcriptionally regulate many differentiation-specific genes, including K1 and K10. Additionally, Notch collaborates with AP-2 TFs to fully initiate spinous cell differentiation (Moriyama et al., 2008; Wang et al., 2008).

2.2.3.2. TF functions in terminal differentiation

Despite the clustering of EDC genes, a local master regulator has not been identified. Thus, it appears that EDC genes are controlled by a pool of ubiquitously expressed TFs. They include Klf4, GATA3, Grainyhead-like 3 (Grhl3), and Aryl hydrocarbon receptor nuclear translocator (Arnt). These factors are crucial for terminal epidermal differentiation, and their ablation in mice is lethal due to reduced expression of EDC genes and consequently skin barrier defects (de Guzman Strong et al., 2006; Geng et al., 2006; Jaubert et al., 2003; Patel et al., 2006; Segre et al., 1999; Ting et al., 2005).

2.2.3.3. Epigenetic regulation of epidermal differentiation

The involvement of polycomb group proteins Bmi1, Cbx4, Ezh2 and Jarid2, as well as additional epigenetic modifiers in establishing tissue-specific differentiation programs in the epidermis has been demonstrated recently (Aarenstrup et al., 2008; Driskell et al., 2012; Ezhkova et al., 2011; Ezhkova et al., 2009; Fessing et al., 2011; Kashiwagi et al., 2007; Luis et al., 2011; Mejetta et al., 2011).

In the epidermis, loss of Ezh2 impaired proliferation and induced premature differentiation of keratinocytes during embryonic development. Mechanistically, it was proposed that EDC gene expression was due to increased AP-1 TF activity in the absence of the repressive H3K27me3 mark (Ezhkova et al., 2009). Interestingly, despite the complete absence of H3K27me3 marks in Ezh1/2 null keratinocytes, only few genes bound by PRC2 were actually up-regulated in the absence of Ezh1/2, suggesting that the repression of differentiation genes is not only mediated by trimethylation of H3 at K27 (Ezhkova et al., 2011).

2.3. Dermal and epidermal cross-talk

Transcriptional mechanisms in the skin are also influenced by interactions between mesenchymal, hematopoietic and epithelial cells, which are responsible for complex events such as tissue development, homeostasis, repair as well as disease (Werner and Smola, 2001). In mammalian skin, multiple types of resident cells are required to create a functional tissue and support tissue homeostasis and regeneration. Pioneering studies of Rheinwald and Green demonstrated that epidermal keratinocytes depend on the presence of fibroblasts for efficient growth in tissue culture (Rheinwald and Green, 1975). Later on, a fibroblast-derived growth factor (FGF), termed Keratinocyte Growth Factor (KGF), which strongly simulated the proliferation of keratinocytes, was identified (Finch et al., 1989). Disrupting the expression of these factors in fibroblasts severely affected the proliferation

and differentiation of overlying keratinocytes (Szabowski et al., 2000).

During development, the epidermal-dermal interactions coordinate hair follicle (HF) formation. The first recognizable feature of HF morphogenesis is the formation of the placode, an epidermal invagination that occurs in the epidermis at the site of an underlying dermal condensation of mesenchymal cells (Fuchs, 2007; Hardy, 1992). Intraepidermal Wnt signaling is necessary and sufficient for HF initiation (Fu and Hsu, 2013). Furthermore, two recent studies unravel important functions of intradermal adipocytes and macrophages in follicular stem cell activation and HF fate, underlining the importance of epidermal-dermal interaction in HF cycling (Castellana et al., 2014; Festa et al., 2011).

2.4. Skin diseases

Deregulation of the described gene expression programs during epidermal stratification as well as deregulation of the dermal and epidermal crosstalk can cause a broad range of skin diseases. The frequent incidence of human disorders characterized by aberrant epidermal differentiation and consequently skin barrier defects, such as Atopic Dermatitis (AD) and Psoriasis, and epidermal cancers, that affect more than 25% of the general population, highlight the importance of coordinated transcriptional programs in keratinocytes (Blanpain et al., 2007; de Cid et al., 2009; Deady et al., 2014; Rogers et al.; Smith and Barker, 2006). Keratinocytes are a source of stress-associated cytokines and chemokines produced in response to mechanical insults, cutaneous pathogens or oncogenic transformation (Mullins et al., 2009; Wollina et al., 2004). Because they constitute the majority of cells in the epidermis, the skin's primary barrier, they are the first cell type to be exposed to harmful insults and respond rapidly by secreting soluble factors and increasing their proliferative potential in order to restore tissue damage (Gause et al., 2013; Hong et al., 2001; Nagao et al., 2012).

Since the skin is a multifaceted organ composed of several cell types, the etiology of skin diseases involves a complex crosstalk among epidermal keratinocytes, dermal fibroblasts, endothelial cells and the immune system. The production of cytokines, chemokines and growth factors is thought to mediate these diseases (Wagner et al., 2010).

2.4.1. Inflammatory skin diseases

AD and psoriasis are the two most common chronic inflammatory skin diseases found in the general population characterized by dry and scaly skin (Holgate, 1999). Although both diseases are generally regarded as immune-mediated conditions, genetic studies have indicated the importance of abnormalities in epithelium-expressed genes as a primary

cause. Loss of function alleles of the skin barrier protein Flg were found to be a major predisposing factor for AD (Palmer et al., 2006) and a copy number polymorphism of a beta defensin gene cluster was associated with increased risk for psoriasis (Hollox et al., 2008). The histological abnormalities of skin biopsies of patients with AD and psoriasis include excessive keratinocyte proliferation, expression of specific keratins, such as K6 and K16 as well as impaired differentiation of epidermal keratinocytes. Disturbed keratinocyte differentiation results in a diminished thickness of the stratum granulosum of the epidermis and an irregular and thickened *stratum corneum* (hyperkeratosis) with retention of nuclei within corneocytes (parakeratosis). Chronic dermatitis, with infiltration of immune cells into the dermis and subcutaneous tissue, and abnormal development of blood vessels are also characteristic features of AD and psoriasis (Nedoszytko et al., 2014). Even though lesional skin of both patients with AD and psoriasis is heavily infiltrated with activated T cells that produce inflammatory cytokines, the mechanisms for skin inflammation and the propensity for skin infections are quite different in the two diseases (Nomura et al., 2003a; Nomura et al., 2003b). The immune response in AD is Th (T helper cell) 2-mediated contributing to the high IgE levels as well as eosinophil and mast cell infiltration, characteristic of this condition (Leung, 2000). Additionally, the secretion of the cytokine Thymic Stromal Lymphopoietin (TSLP) by keratinocytes has been linked to AD (Ziegler and Artis, 2010). High Th2-cytokines, such as IL-4, and low levels of proinflammatory cytokines such as IFN-γ and TNF-α produced by AD skin might explain the increased susceptibility of AD patients to skin infections by microorganisms (Nomura et al., 2003b).

In contrast, the immune response in psoriasis is Th1/Th17-mediated and associated with local neutrophil and macrophage infiltration (Di Cesare et al., 2009; Nickoloff, 2001). Psoriatic keratinocytes produce proinflammatory cytokines, such as IL-1β, IFN-γ and TNF-α (Johnson-Huang et al., 2009) as well as antimicrobial β-defensins and neutrophil-chemoattractant S100 proteins (Lowes et al., 2007).

In both inflammatory skin diseases, the production of these secreted factors by keratinocytes contribute to dendritic cell (DC) activation (Soumelis et al., 2002). Once activated, DCs migrate into skin-draining lymph nodes to present antigens to naïve CD4+ and CD8+ T cells and promote their differentiation into Th1/Th17 or Th2 cells. Attracted by keratinocyte-derived factors, inflammatory cells migrate into the skin via lymphatic and blood vessels (Johnson-Huang et al., 2009).

2.4.1.1. Treatment of inflammatory skin diseases

A multitude of topical and systemic treatments, including glucocorticoids, retinoids, cyclosporin and ultraviolet irradiation have been used for the treatment of psoriasis and mild AD (Bieber, 2008; Wagner et al., 2010). In addition, biologic agents that target either general inflammatory pathways, such as the TNF pathway for psoriasis (Menter et al., 2009), or distinct pathways of immune cell activation, such as antibodies or inhibitors that block IL-4, IL-13, IL-17 and IL-23, have become part of the therapeutic strategies for AD and psoriasis patients (Beck et al., 2014; Ratner, 2015; Yiu and Warren, 2015). Despite progress in the management of these diseases, patients with moderate to severe AD disease still poorly respond to treatment and psoriasis patients usually relapse (Beck et al., 2014). Therefore, it is necessary to develop both improved and novel curative strategies for the treatment of AD and psoriasis (Wagner et al., 2010).

2.4.2. Skin cancer

Skin cancer is the third most common human malignancy and its occurrence has been increasing rapidly over the past decades. An estimated number of 2-3 million non-melanoma skin cancer patients and 132,000 patients of melanoma are counted every year (World health organization).

Melanoma is the type of skin cancer that arises from melanocytes, melanin-producing cells located in the basal layer of the epidermis. It is the most dangerous type of skin cancer as it is the leading cause of death from skin disease (Tsao et al., 2012).

Non-melanoma skin cancer comprises two major types of skin cancers, Basal and Squamous Cell Carinoma (BCC and SCC). Both tumors arise from keratinocytes, but are very different in morphology and in the underlying mechanisms (Colmont et al., 2012).

2.4.2.1. Basal cell carcinoma (BCC)

BCC is the most common and least dangerous form of skin cancer (Kasper et al., 2012).

Several genes of the Sonic Hedgehog (SHH) signaling pathway are frequently mutated in BCCs. Approximately 90% of sporadic BCCs have identifiable mutations in at least one allele of *PTCH1*, and an additional 10% have activating mutations in the downstream effector protein smoothened (SMO), which renders SMO resistant to inhibition by PTCH1 (Epstein, 2008). Several compounds targeting members of the SHH signaling pathway are currently used in clinical trials. Removal of tumors using surgery in less invasive BCCs as well as topical treatment with the Toll-like receptor (TLR) 7/8 agonists Resiquimod or Imiquimod are widely established (Kasper et al., 2012).

2.4.2.2. Cutaneous Squamous cell carcinoma (SCC)

SCCs are the second most common type of human cancer with over 250,000 new cases annually in the USA and it is the second in incidence after BCC. It arises from keratinocytes of the epidermis and oral mucosa. Unlike BCCs, cutaneous SCCs are associated with substantial risk of metastasis and are notoriously resistant to conventional and targeted drug treatments. SCC typically manifests as a spectrum of progressively advanced malignancies, ranging from a precursor lesion like actinic keratosis (AK) to SCC *in situ*, invasive SCC and finally metastatic SCC (Ratushny et al., 2012)

Similar to other cancers, SCCs exhibit impaired genomic stability that facilitates acquisition of new mutations. p53 is commonly mutated in dysplasic lesions. 40% of SCCs *in situ* harbor p53 mutations, indicating that p53 loss occurs prior to tumor invasion (Campbell et al., 1993; Pierceall et al., 1991b). Aberrant activation of Epidermal Growth Factor Receptor (EGFR) and Fyn, a Src-family tyrosine kinase, are found in human SCCs. Furthermore, amplification and activating mutations of the *Ras* oncogene have been described in SCCs (Pierceall et al., 1991a).

Skin SCC has been extensively modeled by either making use of genetically modified mice, such as K14-HPV (Human Papilloma Virus) or K5-SOS-F mouse models (Arbeit et al., 1994; Sibilia et al., 2000) or by using a two-step chemical carcinogenesis protocol. In this protocol, mutations in H-Ras are induced by a single topical dose of a carcinogen, most commonly 7,12-dimethyl-benz[a]anthracene (DMBA), applied on the back skin. Repeated topical applications of a tumor promoter, such as TPA give rise to benign neoplastic lesions, characterized by sustained hyperplasia (papillomas) and inflammation. A small percentage of these papillomas progress to malignant invasive SCCs. In these carcinomas, loss of heterozygosity (LOH) and mutations of p53 are frequent, similar to human SCC (Brown et al., 1986; Quintanilla et al., 1986).

2.4.2.3. Tumor microenvironment in skin cancers

The tumor microenvironment/stroma is composed of leukocytes, fibroblasts (cancer associated fibroblasts), endothelial cells, soluble factors (cytokines, chemokines, matrix metalloproteases, growth factors) and ECM (Hanahan and Weinberg, 2011). Interestingly, stroma shows many features of the skin wound healing response after tissue damage. In contrast to wounds, however, in cancers this process is chronically perturbed, resulting in uncontrolled cell proliferation, invasive growth and eventually metastasis (Dvorak, 1986; Schafer and Werner, 2008).

It is well established that inflammatory processes can both hinder and facilitate cancer by fostering infiltration of leukocytes and promoting stromal remodeling (Demaria et al., 2010). In human skin, the development of AK lesions and *in situ* SCC is linked to chronic inflammation. These lesions can be effectively reversed by treatment with TLR agonists that trigger a potent acute inflammatory reaction, which results in tumor stasis/clearance within a few weeks (Ibrahim and Brown, 2009). Additionally, the risk of SCC is substantially increased in clinical conditions associated with chronic inflammation. However, not all types of inflammation increase the cancer risk as reflected by the surprising skin cancer resistance of psoriasis patients (Nestle et al., 2009).

Interestingly, two recent studies highlight the importance of the keratinocyte-derived cytokine TSLP on inducing tumor protective inflammation by activating CD4+ and CD8+ T cells. Using mouse models of impaired Notch signaling in the skin, which develop epidermal barrier defects and AD-like skin inflammation, it was demonstrated that abrogation of T cell-mediated protection results in the accumulation of tumor-promoting $CD11b^{+}Gr1^{+}$ myeloid cells, which secrete Wnt ligands and thereby augment β-catenin signaling in the neighboring epithelium **(Figure 6-I)** (Demehri et al., 2012; Di Piazza et al., 2012).

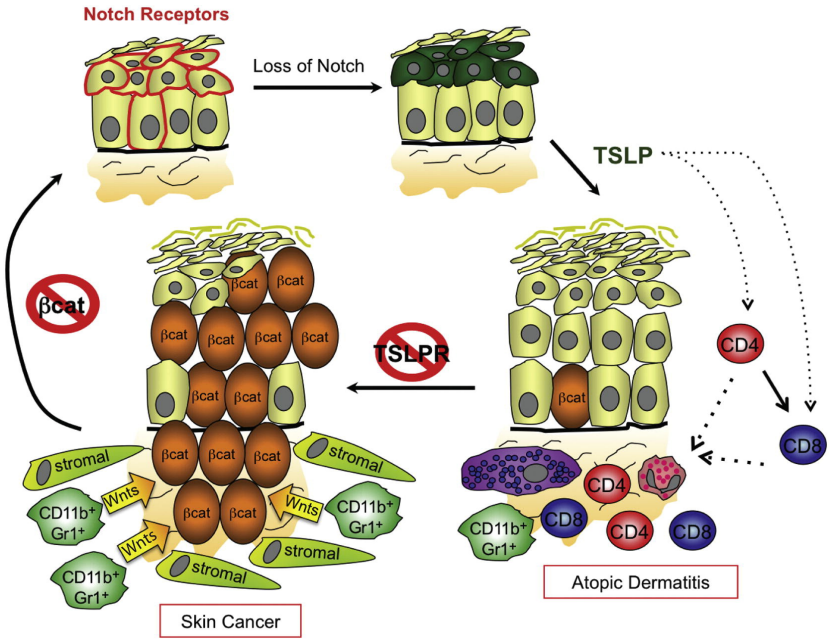


Figure 6-I. TSLP protects from skin cancer by directly activating dermal T cells. Skin tumor formation upon blockade of TSLP. Tumors recruit Wnt-secreting protumorigenic $CD11b^{+}Gr1^{+}$ cells. The development of skin tumors is β-catenin dependent. *Link: Skin barrier defects - epidermal TSLP secretion – skin cancer by Di Piazza et al., 2012.*

Collectively, these studies underline the important functions of transcriptional regulators in epidermal differentiation and demonstrate that a perturbation in this process can result in tumor-protective skin inflammation controlled by a keratinocyte-derived cytokine, which acts on specific T cell subsets. Additionally, these studies reveal that different subsets of immune cells mediate the outcome of skin inflammation from tumor protective to tumor promoting.

2.4.3. AP-1 functions in epidermal homeostasis and disease

Deregulated AP-1 expression is found in skin biopsies of patients with inflammatory/proliferative skin diseases, such as psoriasis and epithelial cancers, such as SCCs (Briso et al., 2013; Eckert et al., 2013; Guinea-Viniegra et al., 2014; Guinea-Viniegra et al., 2012; Zenz et al., 2005). Interestingly, genome scans revealed AP-1 consensus binding sites throughout the EDC cluster (Ezhkova et al., 2009), suggesting regulatory functions of AP-1 TFs on these genes.

The generation of genetically engineered mouse models (GEMMs) has provided important insights into the functions of Jun proteins as well as c-Fos in epidermal homeostasis, inflammation and cancer. More specifically, primary mouse keratinocytes proliferate poorly and show increased expression of early differentiation markers, such as K10 in the absence of c-Jun. Furthermore, the epidermal deletion of c-Jun in tumor-prone *K5-SOS-F* transgenic mice resulted in smaller papillomas, with reduced expression of EGFR (Epidermal Growth Factor Receptor) in basal keratinocytes (Zenz et al., 2003). Notably, mutant mice with epidermal-specific deletion of JunB develop a G-CSF-dependent inflammatory skin disease (Meixner et al., 2008). Additionally, an important function of skin-derived IL-17 on osteoblast differentiation has been characterized in this model (Uluçkan and Wagner, unpublished). Deletion of both JunB and c-Jun, in a constitutive or inducible manner, leads to perinatal death of newborn pups and to a psoriasis-like disease in adults, respectively, in which TNFα and the TIMP-3/TACE pathway as well as C3 and S100A8/A9 signaling play central roles (Guinea-Viniegra et al., 2009; Schonthaler et al., 2014; Zenz et al., 2005). Recent results showed that c-Fos, which regulates keratinocyte differentiation under oncogenic stress conditions (Guinea-Viniegra et al., 2012), acts as a proto-oncogene in the skin and promotes the formation of SCC (Briso et al., 2013). In contrast, the functions of the Fos-related proteins Fra-1 and Fra-2 in the epidermis are still poorly understood. As c-Fos is only expressed in keratinocytes upon stress conditions, we speculate that Fra proteins heterodimerize with Jun proteins in keratinocytes to regulate

epidermal homeostasis. Here, the functions of Fra-2 in the epidermis were investigated.

2.4.3.1. Fra-2 functions in physiology and disease

Using gain and loss of function mouse models, our laboratory has previously unraveled important roles of Fra-2 in mesenchymal and hematopoietic cells. It was shown that Fra-2 has essential functions in bone homeostasis as mice lacking Fra-2 display deficiencies in chondrocytes which resulted in less mineralized bone (Karreth et al., 2004). Furthermore, Fra-2 controls osteoclast survival and size through LIF/LIF-receptor signaling and hypoxia. Fra-2-deficient mice have giant osteoclasts and the expression of HIF-1α and Bcl-2 is increased (Bozec et al., 2008). More recent studies revealed an additional function of Fra-2 as positive regulator of bone formation, since its deletion results in osteoblast differentiation defects, whereas over-expression of Fra-2 revealed an osteosclerotic phenotype (Bozec et al., 2010). In addition, mice lacking Fra-2 in osteoblasts exhibit increased body fat and glucose intolerance with increased adiponectin expression (Bozec et al., 2013).

Notably, Fra-2 transgenic mice, ectopically expressing Fra-2 in various organs, develop generalized fibrosis with predominant manifestation in the lung and the liver (Eferl et al., 2008). Recent data from our lab indicate that Fra-2 in mesenchymal cells of the lung and the liver might be relevant in fibrosis.

In mouse skin, Fra-2 is expressed in all epidermal layers (Eckert et al., 2013). The Fos-related protein is known to be an ERK 1/2 MAP kinase target (Eriksson and Leppa, 2002; Murakami et al., 1997) and the ERK 1/2 MAP kinase pathway is active during Ca^{2+} induced differentiation via PKCα (Schmidt et al., 2000; Seo et al., 2004). Therefore, the functions of Fra-2 during keratinocyte differentiation and skin homeostasis were investigated.

Objectives

Objectives

AP-1 transcription factors have important functions in skin development, inflammation and cancer. However, the functions of the Fos-related protein Fra-2, an important regulator of mesenchymal and hematopoietic cell fate, have not been studied in epithelial cells.
The following objectives were addressed in this thesis:

1.) Investigate the functions of Fra-2 during keratinocyte differentiation. More specifically, analyze the expression, post-translational modifications and identify novel interaction partners and target genes of Fra-2 *in vitro* during calcium-induced keratinocyte differentiation.

2.) Generate epithelial-specific gain- and loss-of-function mouse models of Fra-2 to validate the expression of the identified target genes, and to uncover deregulated pathways that may interfere with epidermal homeostasis, affect inflammatory processes and eventually lead to skin cancer development.

3.) Dissect the molecular mechanisms of how aberrant epidermal differentiation, skin inflammation and skin cancer formation are linked using these genetically engineered mouse models, skin transplantation models and primary keratinocyte cultures.

Objectivos

Objectivos

Los factores de transcripción AP-1 desempeñan funciones importantes en el desarrollo de la piel, los procesos inflamatorios y el cáncer. Sin embargo, las funciones de la proteína de la familia de Fos Fra-2, un importante regulador del destino final de células hematopoyéticas y mesenquimales, no se han estudiado en células epiteliales. Por lo tanto, se abordaron los siguientes objetivos en esta tesis:

1.) Investigar las funciones de Fra-2 durante la diferenciación de los queratinocitos. Más específicamente, analizar la expresión, las modificaciones post-traduccionales e identificar nuevas moléculas de interacción y genes diana de Fra-2, *in vitro,* durante la diferenciación de queratinocitos inducida por calcio.

2.) Generar modelos de ratón con ganancia o pérdida de función de Fra-2 específicamente en células epiteliales para validar la expresión de los genes diana identificados, y para descubrir vías desreguladas que pueden interferir con la homeostasis epidérmica, afectar a los procesos inflamatorios y eventualmente resultar en el desarrollo de cáncer de piel.

3.) Diseccionar los mecanismos moleculares de cómo los procesos de diferenciación epidérmica aberrante, la inflamación de la piel y la aparición de cáncer de piel están vinculados utilizando estos modelos de ratones genéticamente modificados, modelos de trasplante de piel y cultivos de queratinocitos primarios.

Materials and Methods

1. MICE

1.1. Study approval

All experiments were performed in accordance with institutional policies and national and European guidelines. Mice were kept in the CNIO animal facility and all animal experiments were approved by the Animal Experimental Ethics Committee of the Instituto de Salud Carlos III (Madrid, Spain).

1.2. Generation of Fra-2 and Fra-2/p65 loss-of-function mouse models

To generate epithelial-specific Fra-2 loss-of-function (LOF) mutants and Fra-2/p65 double knock-out (DKO) mutants, the Cre-loxp system was used (Orban et al., 1992).

Fra-2$^{\Delta sb}$ mice were generated by crossing mice carrying Fra-2 alleles flanked by loxP sites (*Fra-2*$^{f/f}$) (Eferl et al., 2007) with mice expressing Cre recombinase under the control of the FoxN1 promoter (Soza-Ried et al., 2008). *Fra-2*$^{\Delta ep}$ mice were generated by crossing *Fra-2*$^{f/f}$ mice with mice expressing Cre recombinase under the control of the K5 promoter (Tarutani et al., 1997). *Fra-2/p65*$^{\Delta ep}$ mice were generated by crossing *Fra-2*$^{f/f}$ mice and *p65*$^{f/f}$ mice (Algul et al., 2007) with mice expressing Cre recombinase under the control of the K5 promoter. Control (Cre negative mice), *Fra-2*$^{\Delta sb}$ mutants (*Fra-2*$^{f/f}$*; FoxN1 Cre*$^{+/T}$), *Fra-2*$^{\Delta ep}$ mutants (*Fra-2*$^{f/f}$*; K5 Cre*$^{+/T}$) and *Fra-2/p65*$^{\Delta ep}$ mutants (*Fra-2*$^{f/f}$*; p65*$^{f/f}$*; K5 Cre*$^{+/T}$) were identified by genotyping PCR on tail biopsies using standard PCR conditions and the following primers **(Table 1-M)**.

Table 1-M

Allele Name	Primer	Sequence (5' – 3')
Fra-2(f/+)	sense	GAGGGAGTTGGGGATAGAGTGGTA
	antisense	GGACAGCAGGTCAGGAGTAGATGA
Fra-2Δ	sense	GAGGGAGTTGGGGATAGAGTGGTA
	antisense	TGTACCGGACGCTTGTCATCTCAT
p65(f/+)	sense	GAGCGCATGCCTAGCACCAG
	antisense	GTGCACTGCATGCGTGCAG
p65Δ	sense	GTGCACTGCATGCGTGCAG
	antisense	ACTGAAGCGGCCAGGATG
Cre	sense	GCATTACCGGTCGATGCAACGAGTGATGAG
	antisense	GAGTGAACGAACCTGGTCGAAATCAGTGCG

1.3. Generation of Fra-2 gain-of-function mouse model

To generate an epithelial-specific Fra-2 gain-of-function (GOF) mouse model with inducible expression of Fra-2, we made use of the Tretracycline-Controlled-Transcriptional Activation (tTA) method. This system permits the switchable expression of a specific gene upon the presence or absence of the tetracycline antibiotic or one of its derivatives such as doxycycline (Gossen and Bujard, 1992)

Fra-2$^{Ep\text{-}tetOFF}$ mice were generated by crossing mice carrying a Flag-tagged *Fra-2* cDNA knock-in controlled by a tetracycline operator, downstream of the *col1a1* gene (*col Fra-2*$^{+/KI}$) (Bozec et al., 2013) to mice expressing *tTA* from the K5 promoter (Diamond et al., 2000). Control (*col Fra-2*$^{+/KI}$*; K5tTA*$^{+/+}$) and *Fra-2*$^{Ep\text{-}tetOFF}$ mutants (*col Fra-2*$^{+/KI}$*; K5tTA*$^{+/T}$) were identified by genotyping PCR on tail biopsies using standard PCR conditions and the following primer sequences **(Table 2-M)**.

Table 2-M

Allele Name	Primer	Sequence (5' – 3')
col Fra-2	sense	GCACAGCATTGCGGACATGC
	antisense 1	CCCTCCATGTGTGACCAAGG
	antisense 2	GCAGAAGCGCGGCCGTCTGG
K5tTA	sense	ATGCTACCATAGTCATATCGTCATGCATGCAT
	antisense	TATCGTCATGCATGCATATGCTACCATAGTCA

Fra-2$^{Ep\text{-}tetOFF}$ *SOS+* mice were generated by crossing *Fra-2*$^{Ep\text{-}tetOFF}$ mice to *K5-SOS-F* mice (*EGFR*$^{wa/wa}$*, K5 SOS*$^{+/T}$) (Sibilia et al., 2000). *EGFR*$^{wa/wa}$ and *K5 SOS*$^{+/T}$ genotypes were evaluated by macroscopic appearance and used to generate *SOS+* (*col Fra-2*$^{+/KI}$*, K5tTA*$^{+/+}$, *EGFR*$^{wa/+}$*, SOS*$^{+/T}$) and *Fra-2*$^{Ep\text{-}tetOFF}$ *SOS+* (*col Fra-2*$^{+/KI}$*, K5tTA*$^{+/T}$*, EGFR*$^{wa/+}$*, SOS*$^{+/T}$) mice. All mice generated were maintained in the absence of doxycycline so that transgene expression was turned on during embryonic development.

1.4. Dye penetration assay

Timed matings were set up, a positive vaginal plug in the early morning was considered as E0.5 and pregnant females were sacrifized at E17.5 to collect embryos. E17.5 embryos were rinsed once in PBS and once in 100% methanol, stained in 0.1% Toluidine blue for 30 minutes at room temperature and washed in PBS before image capture.

1.5. Mouse to mouse skin grafts

Back skin of *Fra-2*$^{\Delta ep}$ and *Co* pups was dissected at postnatal day 2 (P2), cut longitudinally into two parts of equal size and grafted onto 4-8 week old Nod/SCID gamma mice by stitching with Premilene 6/0. Skin grafts were wrapped and hosts were treated with Ibuprofen for 24h after transplantation and antibiotics for 2 weeks after transplantation. Wrapping was changed one week after transplantation and removed two weeks after transplantation. The graft on the immuno-deficient mouse was followed over 4 weeks.

2. HISTOLOGY

2.1. Formalin-fixed paraffin-embedded tissue

Back skins from embryos at embryonic day E17.5, back skins from pups at postnatal day 6 (P6) and tails skins from 1-month old adult mice were fixed overnight (o/n) with buffered 10% PFA at 4°C and embedded in paraffin. 3µm skin sections were cut. Hematoxylin and Eosin (H&E) and Immunohistochemistry (IHC) were performed as previously described (Zenz et al., 2005). Antigen retrieval was performed in sodium citrate (10mM, pH 6) in a pressure cooker, slides were blocked in PBS, 0.1% Triton X, 1%BSA, 1% donkey serum and washed in PBS, 0.1% Triton X. The following primary antibodies (Abs) were used for IHC: p-ERK1/2 (Thr202/Tyr204) (1:1000, #4370, Cell Signaling), Fra-2 (1:50, CNIO Monoclonal Antibody Unit), CD45 (1:200, BD Pharmingen 550539), Ki67 (1:200, Dako) and p65 (1:1000, Santa Cruz sc-372). Elite ABC kit (Vector Labs) secondary Abs were used and nuclei were counterstained with hematoxilin. Sections were analyzed by light microscopy (LeicaDM2500).

2.2. Fresh frozen tissue

Back skins from embryos at E17.5 and tail skins from adult mice were embedded in optimal cutting temperature (OCT) compound, snap frozen in dry ice and cut at 10µm. Immunofluorescence (IF) was performed as described previously (Perez-Moreno et al., 2006). Tissues were fixed in 4% PFA, slides were blocked in PBS, 0.1% Triton X, 1%BSA, 1% donkey serum, washed in PBS, 0.1% Triton X. Primary antibodies used for IF were: Fra-2 (1:50, CNIO Monoclonal Antibody Unit), K5 (1:1000, Covance), Filaggrin (1:1000, Covance), Loricrin (1:1000, Covance), Ki67 (1:200, Dako), Ezh2 (1:1000, #5246, Cell Signaling). Alexa Fluor secondary Abs conjugated to 488nm or 555nm emitting fluorophores (Invitrogen) were used. Sections were mounted with Mowiol + DAPI (Vectashield) and analysed by confocal microscopy.

3. MUTAGENESIS AND CLONING

For the generation of Fra-2 methyl- and phospho-mutants, site-directed mutagenesis was carried out on Flag-tagged mouse Fra-2 cloned in pGEM7Zf vector using the QuikChange Lightening Site-directed Mutagenesis kit (Stratagene, Agilent technologies) following the manufacturer's instructions. The following primers were used to generate the indicated point mutations **(Table 3-M)**.

Table 3-M

Mutation	Primer	Sequence (5' – 3')
K104F	sense	CACGGTGGTACCGATGGTAAAGATCACTCCAGGTCTGG
	antisense	CCCAGACCTGGAGTGATCRRRACCATCGGTACCACCGTG
S320A/T322A	sense	CAGGGCTAGAAGTGCGGGGGCGTTCAAGGAGTCTG
	antisense	CAGACTCCTTGAACGCCCCCGCATTCTAGCCCTG

After sequencing, Flag-tagged *Fra-2 WT*, *Fra-2 K104F* and *Fra-2 S320A/T322A* were subsequently cloned into pLVX for lentiviral expression. Briefly, 5µg of plasmid DNA of pGEM7Zf containing Flag-tagged *Fra-2 WT*, *Fra-2 K104F* or *Fra-2 S320A/T322A* inserts and 1µg of plasmid DNA of pLVX was digested with the restriction enzymes EcoRI and XhoI (New England Biolabs). Digested pLVX vector was treated with shrimp alkaline phosphatase to avoid re-ligation. Digested pGEM7Zf plasmid DNA was separated on a 1% agarose gel and insert DNA was purified using QIAquick Gel Extraction Kit (Qiagen). Ligation was performed o/n at RT using T4 DNA ligase (Fermentas). Competent bacteria (Invitrogen) were transformed by heat shock transformation and plated on Luria Broth (LB) plates containing ampicillin and were incubated o/n at 37°C. Eight clones were collected from each transformation and grown in 2ml LB media containing ampicillin o/n. Plasmid DNA was isolated using QIAprep Spin Miniprep kit (Qiagen). Presence and correct orientation of the insert was confirmed by restriction digest with XhoI and XbaI (New England Biolabs) and subsequent sequencing.

4. CELL CULTURE

4.1. Mouse keratinocyte cell lines

For long term culture, mouse keratinocyte (mKC) cell lines were isolated from back skin of newborn mice as previously described (Nowak and Fuchs, 2009). Briefly, the epidermis was separated from the dermis by incubation in 0.75% trypsin (Gibco) at 37°C for 1h.

Single epidermal cells were isolated by incubation in media containing 250µg/ml DNaseI (Sigma) in a 37°C water bath with frequent shaking. The cell suspension was filtered through a 70µm cell strainer. mKCs were cultured on feeders in DMEM (Sigma) media containing 0.05mM calcium (Ca^{2+}), L-Glutamine (Gibco), Penicillin, Streptomycin (Pen/Strep; Gibco), 15% chelated fetal bovine serum (FBS; Lonza; supplemented with insulin, cholera toxin, hydrocortisone, transferrin and triiodo-L-thyronine) until they became feeder-independent. All experiments were performed with feeder-independent mKCs up to passage 20. To induce differentiation in culture, Ca^{2+} was raised from 0.05mM to 1.5mM for the indicated time periods. Ezh2 methyltransferase activity was inhibited using GSK126 (GlaxoSmithKline) and EPZ6438 (Epizyme) inhibitors at 5µM for 6-48h. ERK1/2 kinase activity was inhibited using FR180204 (Tocris Bioscience) at 5µM for up to 48h.

4.2. Primary mouse keratinocyte culture

Isolation and short term culture of primary mKCs from tail skin of adult wildtype, *Fra-2*$^{f/f}$ mice, *Fra-2*$^{Ep\text{-}tetOFF}$ mice and littermate controls was performed as described (Blanpain et al., 2004). Briefly, single epidermal cells were isolated as stated above. mKCs were plated on coated dishes (Cascade Biologics) in DMEM (Sigma) media containing 8% chelated FBS (Lonza), 0.05mM Ca^{2+} and Pen/Strep. Media was changed to serum-free keratinocyte medium (K-SFM Gibco) supplemented with EGF, bovine pituitary extract and Pen/Strep 12h after plating. *Fra-2*$^{Ep\text{-}tetOFF}$ keratinocytes were cultured in the absence of doxycycline. Calcium concentration was raised to 1,5mM for differentiation experiments.

Fra-2$^{f/f}$ cells were infected with adenoviruses expressing Cre (*AdCre*), green fluorescent protein (*AdGFP*) or an empty adenovirus (*Ad0*) purchased from the University of Iowa. 300 particles per cell were used on day 1 and 4 after plating. Cells were collected for mRNA and protein analyses 7 days after plating.

4.3. Lentiviral production

Lentiviruses were produced in 293T cells (cultured in DMEM + 10% FBS and Pen/Strep) by HEPES-buffered saline–$CaCl_2$ phosphate-mediated transient co-transfection of pLVX plasmids and pLP1, pLP2, pLP/VSVG packaging plasmids. Primary *WT* cells were infected with lentiviruses on day 1 and 2 after plating. Primary *Fra-2*$^{f/f}$ cells were infected with *AdGFP/AdCre* viruses on day 1 and day 4 and with lentiviruses on day 2 and day 3 after plating. Cells were collected for mRNA and protein analyses 5 days after the last lentiviral infection.

4.4. Feeder cells

3T3 fibroblasts were used as feeders for mKCs and were maintained in 10% FBS DMEM 3:1 F12 (Gibco) with sodium bicarbonate, L-glutamine and Pen/Strep. These cells were passaged up to 20 times. Feeders were treated with 10µg/ml mitomycin C for 2h at 37°C and washed extensively before mKCs were plated on top.

5. PROTEIN ANALYSES

5.1. Protein Isolation

Protein isolation from epidermal tissue or from cultured cells for western blot and IP was performed in RIPA buffer (50mM Tris HCl pH7.5, 150mM NaCl, 5mM EDTA, 5mM EGTA, 1% NP-40) containing protease and phosphatase inhibitors (Roche). Cell lysis buffer (#9803 Cell Signaling) was used to isolate protein from epidermal samples for ELISA. Protein lysates were quantified using Pierce BCA protein Assay Reagent (Thermos Scientific).

5.2. Cytokine analyses

BD CBA Flex kits (Beckton-Dickinson) were used to measure IL-6, IL-17, IL-1α, TNFα and G-CSF levels in serum following manufacturers instructions. Serum was diluted 1:4.
A single ELISA kit (R&D Biosciences) was used to measure TSLP levels in epidermal lysates and serum following manufacturers instructions. Serum was diluted 1:4.

5.3. p65/NF-κB transcription factor activity assay

p65 activity was measured in nuclear extracts isolated from epidermis or primary keratinocytes using a DNA-binding ELISA kit (TransAM, Active Motif). Briefly, nuclei were isolated by cell lysis in hypotonic buffer (20mM Hepes, pH 7.5, 5mM NaF, 10µM Na_2MoO_4, 0.1mM EDTA, 0.5% NP-40) and nuclear lysis in complete lysis buffer (provided in the kit). 10µg of nuclear extracts were bound to immobilized oligonucleotides containing the NF-κB consensus site for 1h at RT. After washing, the primary p65 HRP-conjugated antibody was incubated with the bound proteins for 1h at room temperature. After adding the developing solution (provided in the kit), absorbance was read on a spectrophotometer at 450nm.

5.4. Western blots

Western blot (WB) analysis was performed according to standard procedures. Equal amounts of protein (20-50µg) were separated by SDS-PAGE and transferred to

nitrocellulose membranes (Whatman). The following primary Abs were used: Fra-2 (1:100, CNIO Monoclonal Antibody Unit), Ezh2 (1:1000, #AC22 Cell Signaling), p-Fra-1 (Ser265) (1:1000, #3880, Cell Signaling), pan-methyl lysine (1:200, ab174719, Abcam), p-Ser (1:500, P5747, Sigma), p-ERK1/2 (Thr202/Tyr204) (1:1000, #4370), ERK1/2 (1:1000, #4695, Cell Signaling), ΔNp63 (1:500, sc-8609, Santa Cruz), Suz12 (ab12073, Abcam), Flg (1:1000, Covance), Lor (1:1000, Covance), Vinculin (1:1000, V9131, Sigma). Blots were incubated with the appropriate horseradish peroxidase-couples secondary Abs (GE Healthcare) and developed using the Amersham ECL Plus Western Blotting Detection Reagent (GE Healthcare) and Amersham ECL Hyperfilms (GE Healthcare).

5.5. Immunoprecipitation

2-5μg of Fra-2 (CNIO Monoclonal Antibody Unit), Ezh2 (5246, Cell Signaling), and pan-methyl lysine (ab174719, Abcam) antibodies were incubated with 20μl of Dynabead protein G magnetic beads (Invitrogen) for 3h-over night. 1mg of precleared cell extract per immunoprecipitation (IP) was used as input material and incubated with antibody-conjugated beads over night at 4ºC under rotation. A magnetic rack was used to separate beads from protein extracts/buffer.

5.5.1. Immuno-complex elution for WB

IPs were extensively washed with RIPA buffer and captured proteins were eluted from the antibody-coupled beads using Laemmli buffer (125mM Tris-HCl pH 6.8, 20% Glycerol, 4% SDS, 0.005% Bromophenol blue, 10% β-Mercaptoethanol) for Fra-2, methyl lysine and p-Fra-1 western blot analysis or XT sample buffer (Biorad) without β-Mercaptoethanol for Ezh2 western blot analysis.

5.5.2. Immuno-complex elution and digestion for Mass Spectrometry

IPs were extensively washed with RIPA buffer and captured proteins were eluted from the antibody-coupled beads by incubation at 45°C with 200μL of 8M Urea in 100mM Tris-HCl pH 8.0. Protein samples were processed using filter aided sample preparation (FASP) method (Wisniewski et al., 2009b), digested at 25°C overnight using endoproteinase Lys-C (Wako Pure Chemical Industries, Osaka, Japan) at 1:50 enzyme to protein ratio followed by digestion with trypsin (Promega, Madison, WI) at 1:100 enzyme to protein ratio for 6h at 37°C. Peptides were subjected to tip-based strong anion exchange fractionation (SAX) using the described protocol (Wisniewski et al., 2009a). In addition, a second immunoprecipitation experiment was carried out in which the protein eluate was separated

by SDS-PAGE. For in-gel digestion, bands were excised and proteins were reduced with 15mM TCEP in 50mM ammonium bicarbonate for 45 minutes at 60°C and alkylated with 50mM 2-chloroacetamide in 50mM ammonium bicarbonate for 30 minutes at 25°C in the dark. Proteins were subsequently digested at 37°C overnight with trypsin at 1:200 enzyme to protein ratio.

5.6. Mass Spectrometry Analysis

For mass spectrometry (MS) experiments, up to 20mg of precleared protein lysate was used per IP.

5.6.1. LC-MS/MS analysis

Desalted peptides were separated by reversed-phase chromatography using a nanoLC Ultra system (Eksigent), directly coupled with a LTQ-Orbitrap Velos instrument (Thermo Fisher Scientific) via nanoelectrospray source (ProxeonBiosystem). Peptides were loaded onto the column (Dr. Maisch GmbH, Reprosil-Pur C18-AQ 3 µm, 200x0.075 mm), with a previous trapping column step (0.10 x 20 mm, Reprosil-Pur C18-AQ, 3 µm, 120Å), during 10 min with a flow rate of 2.5µL/min of loading buffer (0.1% FA). Elution from the column was made with a 120 min linear gradient or a 45 minutes linear gradient (in-solution and in-gel respectively) (buffer A: 4% ACN, 0.1%FA; buffer B: 100% ACN, 0.1%FA) at 300nL/min. The peptides were directly electrosprayed into the mass spectrometer using a PicoTip emitter (360/20 OD/ID µm tip ID 10 µm, New Objective) a 1.4 kV spray voltage with a heated capillary temperature of 325°C and S-Lens of 60%. Mass spectra were acquired in a data-dependent manner, with an automatic switch between MS and MS/MS scans using a top 15 or a top 5 method (in-solution and in-gel respectively) with a threshold signal of 800 counts. MS spectra were acquired with a resolution of 60000 (FWHM) at 400 m/z in the Orbitrap, scanning a mass range between 350 and 1500m/z. Peptide fragmentation was performed using collision induced dissociation (CID) and fragment ions were detected in the linear ion trap. The normalized collision energy was set to 35%, the Q value to 0.25 and the activation time to 10 ms. The maximum ion injection times for the survey scan and the MS/MS scans were 500 ms and 100 ms respectively and the ion target values were set to 1E6 and 5000, respectively for each scan mode.

5.6.2. Mass Spectrometry data analysis

Raw files were analyzed by Proteome Discoverer (version 1.4.1.14) against a mouse database (Swiss-Prot canonical, 17002 sequences, Jan22_2014 release, including

common contaminant proteins). Oxidation of methionines, monomethylation, dimethylation and trimethylation of lysines were set as variable modifications, whereas carbamidomethylation of cysteines was considered as fixed modification in the SequestHT search engine. Minimal peptide length was set to 6 amino acids, a maximum of two missed-cleavages were allowed. Peptides were filtered at 1% FDR by using Percolator.

6. CHROMATIN IMMUNOPRECIPITATION

Chromatin immunoprecipitation (ChIP) was performed using cultured mouse keratinocytes fixed in 1% formaldehyde. Nuclear lysates were obtained by lysis of cell membranes in a hypotonic buffer (50mM, HEPES-KOH pH 7.5, 140mM NaCl, 1mM EDTA, 10% Glycerol, 0.5% NP-40, 0.25% Triton-X-100) and clearance of detergents (10mM Tris-HCL pH 8.0, 200mM NaCl, 1mM EDTA, 0.5mM EGTA). Nuclei were resuspended in ChIP sonication buffer (100mM NaCl, 10mM TrisHCl pH 8, 1mM EDTA, 0.5mM EGTA, 0.1%Na-Deoxycholate, 0,5% N-lauroylsarcosine). Sonication was performed using a Covaris ultrasound device. Whole nuclear extract was quantified with BCA protein assay and 0.5-1mg of protein was used for preclearing with 10µl of protein G dynabeads. Immunoprecipitation was performed with 2-5µg of antibodies coupled to protein G dynabeads against Fra-2 (CNIO Monoclonal Antibody Unit), Ezh2 (#5246, Cell Signaling), Suz12 (ab12073, Abcam), H3K27me3 (ab6002, Abcam) and H3K4me3 (ab8580, Abcam) over night at 4ºC. Rat or rabbit IgGs were used as a negative control (Millipore). After washing once with low salt (1% Triton X-100, 0.1% SDS, 2mM EDTA pH 8.0, 150mM NaCl, 20mM Tris-HCl pH 8.0), once with high salt buffer (1% Triton X-100, 0.1% SDS, 2mM EDTA pH 8.0, 500mM NaCl, 20mM Tris-HCl pH 8.0), once with LiCl buffer (1% NP-40, 1% Na-Deoxycholate, 1mM EDTA, 250mM LiCl, 20mM Tris-HCl pH8.0), and once with TE Buffer (10mM Tris, 8.0, 1mM EDTA), Protein/DNA complexes were eluted from beads and DNA was decrosslinked at 65ºC in elution buffer (1%SDS, 10mM EDTA, 50mM Tris-HCl pH 8) overnight. Eluate was treated with RNase A (Sigma) for 2h at 37ºC and with Proteinase K (VWR) for 2h at 55ºC. DNA was recovered with Phenol/Chlorophorm extraction and amplified using GoTag PCR master mix (Promega) and primers specific for AP-1 binding sites of EDC promoters using an Ep-Realplex light cycler (Eppendorf). Primer sequences can be found in **Table 4-M**. The position of the AP-1 consensus site upstream the transcription start site is indicated in parentheses.

Table 4-M

Allele Name	Primer	Sequence (5' – 3')
Flg **(-1400bp)**	sense	CCCACTTGCTGAAAAGGTCAA
	antisense	CAGGTTTGGCAGGTGCTGTA
S100A11 **(-200bp)**	sense	GACCTGAACTGCAAATGGACAC
	antisense	CGGTGCTCTAGACTCAGCTC
Lce1k **(-1000bp)**	sense	TCTTAGAAGCTAGGAAGTTTTCCA
	antisense	ACAATGAGAGAAATGCTGCCC
S100A7 **(-3000bp)**	sense	TTTCTGCCCCCTTCTGCAC
	antisense	CCAAACAGGTGGTTCTCATAGG
Lor **(-1400bp)**	sense	ATGACCAACCTGCTCACTGT
	antisense	GAGGGAAGGAGGTGATGCTG
Tchhl1	sense	TGGTGGTGATGAATCAGGTCAT
	antisense	ATGTGGGGCAATGTGGGG
TSLP **(-1200bp)**	sense	GTGTCAACAGCTGGGCCTTTCTTT
	antisense	GTGGGTTGGGTTAAGATTTGCGCT
Intergenic mChr3	sense	TGTCTGGAATGTGGTGGTTTGA
	antisense	GCCCACTGCTATAATTAGGAAGGA

7. FACS

Basal and differentiated mKCs were isolated from the back skins of newborn mice as described above. Back skins from 6 mice were pooled. Cell suspensions were stained for 30 min at 4°C using a phycoerythrin-conjugated rat anti-CD49f (integrin α6 chain) antibody (#555736, BD) as previously described (Nowak and Fuchs, 2009). Cells were sorted on a FACSAria IIu using CellQuest Pro software (BD).

8. RT-qPCR

RNA was isolated from epidermal tissue and cultured cells using Trizol reagent (Invitrogen). cDNA synthesis was performed using 2µg of total RNA using "Ready-to-go You-Prime IT First-Strand-Beads" (Amersham Pharmacia Biotech) and random primers (Invitrogen). RNA was isolated from FACS purified cells using RNeasy Mini kit (Qiagen). cDNA synthesis was performed using 100ng of total RNA using SuperScript VILO cDNA Synthesis Kit. PCR products were quantified using GoTag PCR master mix (Promega),

Ep-Realplex thermal cycler (Eppendorf) and ΔΔCT method. Primer sequences can be found in **Table 5-M**.

Table 5-M

Allele Name	Primer	Sequence (5' – 3')
Flg	sense	GGAGGCATGGTGGAACTGA
	antisense	TGTTTATCTTTTCCCTCACTTCTACATC
S100A11	sense	ACAGCGGGAAGGATGGAAAC
	antisense	TGGAAATCTAGCTGCCCGTC
Lce1k	sense	GGACTGCTGCTGACCTGAGT
	antisense	ACAGAAGGGGTAGCAACATGG
S100A7	sense	TGAAGGGTCCATCAGTCA
	antisense	CTAGTAGAGGCTGTGCT
Lor	sense	TCACTCATCTTCCCTGGTGCTT
	antisense	GTCTTTCCACAACCCACAGGA
Tchhl1	sense	ATTCCGCTTGCTCTCACTCCT
	antisense	GGTTCAACTTTCTCTCCACAGC
TSLP	sense	GAGGACTGTGAGAGCAAGCCAG
	antisense	GGCAGTGGTCATTGAGGGCTT
Fra-2	sense	CGCTCACATCCCTACAGTCC
	antisense	CCTGCAGCTTCTCTGTCAGC
Fra-2 Flag	sense	CTCCCGGCACTTCAAACCT
	antisense	TTGTCGTCGTCGTCCTTGTA
Fra-2ΔGFP	sense	ATCCCGGGAACTTTGACACC
	antisense	GTGAACCGCATCGAGCTGAAGGG
Keratin 1	sense	CTCTACGTAGTTACCACAACAAT
	antisense	CAATCCTCTACGTAGACGTA
S100A8	sense	AGAAGGCCTTGAGCAACCTC
	antisense	TGGCTGTCTTTGTGAGATGC
Lcn2	sense	CAGAAGGCAGCTTTACGATG
	antisense	TCTGATCCAGTAGCGACAGC
GAPDH	sense	GTGTTCCTACCCCCAATGTG
	antisense	GGAGACAACCTGGTCCTCAG

S100A9	sense	GTCCAGGTCCTCCATGATGT
	antisense	TCAGACAAATGGTGGAAGCA
p65	sense	AACCAGGGTGTGTCCATGTC
	antisense	CCGGAGAGACCATTGGGAAG

9. STATISTICAL ANALYSIS

Unless stated differently, all experiments were performed 3 times. Results are presented as mean ± standard deviation (SD) of triplicates. p-values were calculated using two-tailed student's t-test. * indicates $p<0.05$, considered significant.

Results

1. Fra-2 regulates epidermal differentiation

1.1. Fra-2 protein levels are increased during keratinocyte differentiation

To define the role of Fra-2 in epithelial homeostasis, Fra-2 expression was evaluated in cultured mouse keratinocytes (mKCs) in basal conditions and upon calcium (Ca^{2+})-induced differentiation. *Fra-2* mRNA levels did not change during mKC differentiation **(Figure 1A).** However, increased Fra-2 protein was detected upon Ca^{2+} treatment **(Figure 1B)**. *G*ene expression analyses of FACS-purified epidermal keratinocytes obtained from newborn pups further confirmed no difference of *Fra-2* mRNA between basal mKCs (α6 integrin high) and differentiated mKCs (α6 integrin low) **(Figure 1C)**. In mouse skin tissue sections, immunofluorescence (IF) staining revealed that Fra-2 protein was expressed in all epidermal layers. However, a gradient was observed, with highest expression in terminally differentiated keratinocytes **(Figure 1D**, arrows**).**

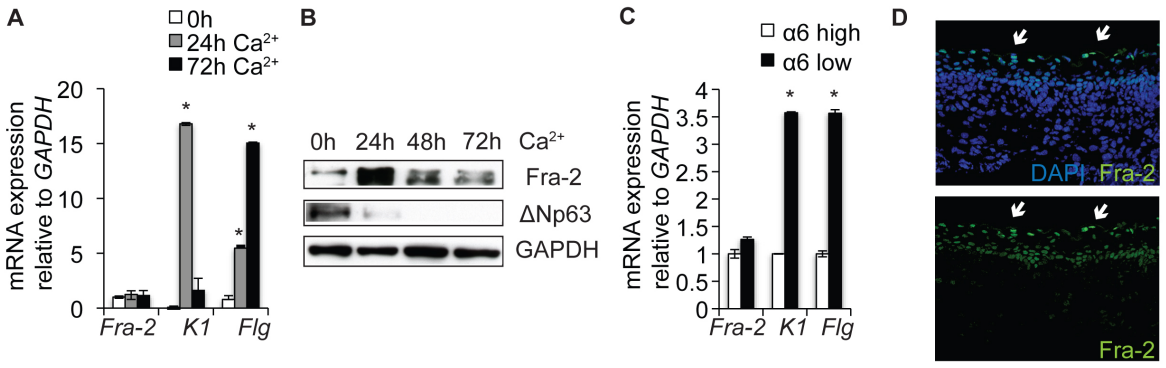


Figure 1. Fra-2 induces terminal epidermal differentiation. (A) *Fra-2* mRNA expression during calcium (Ca^{2+})-induced *in vitro* mouse keratinocyte (mKC) differentiation; *Keratin 1* (*K1*) and *Filaggrin* (*Flg*) expression indicate induction of early and terminal epidermal differentiation markers, respectively; n=3; **(B)** Fra-2 protein expression during Ca^{2+}-induced *in vitro* mKC differentiation; loss of ΔNp63 indicates induction of differentiation; n=4. **(C)** *Fra-2* mRNA expression of basal (α6 integrin high) and differentiated (α6 integrin low) mKCs isolated from newborn pups; *K1* and *Flg* indicate induction of early and terminal epidermal differentiation markers; n=3; **(D)** Fra-2 immunofluorescence (IF) of wild-type skin at embryonic day 17.5 (E17.5); n=6; 40x magnification.
* $p<0.05$; bars represent mean ± standard deviation (SD).

1.2. Fra-2 induces EDC gene expression and binds EDC gene promoters

Based on the increased Fra-2 protein expression during mKC differentiation, a regulatory function of Fra-2 on terminal epidermal differentiation genes was next investigated. For this purpose, primary wild-type (*WT*) mKCs were infected with lentiviruses expressing full length, Flag-tagged, Fra-2 (*Fra-2 WT*) or empty vector (*pLVX*) and the expression of EDC

genes was evaluated. Efficient *Fra-2* over-expression was verified by qRT-PCR. Notably, lentiviral expression of *Fra-2 WT* induced EDC gene expression in primary mKCs **(Figure 2A)**. To test whether Fra-2 directly regulates and binds EDC gene promoters, chromatin immunoprecipitation (ChIP) experiments in basal mKCs and during differentiation were performed. Rat IgGs were used as negative control for Fra-2 ChIP experiments. mRNA from the same samples was collected to confirm epidermal differentiation and to correlate binding with gene expression as shown in **Figure 2B**. Fra-2 associated with EDC promoters in regions with conserved AP-1 consensus sites. Surprisingly, Fra-2 was bound at EDC gene promoters under differentiation conditions, but also in basal conditions, when EDC genes are not expressed **(Figure 2C).** Collectively these findings indicate that Fra-2 induces EDC gene expression by direct promoter binding and suggest that even though expressed and bound at target gene promoters, Fra-2 is likely inactive in basal cells.

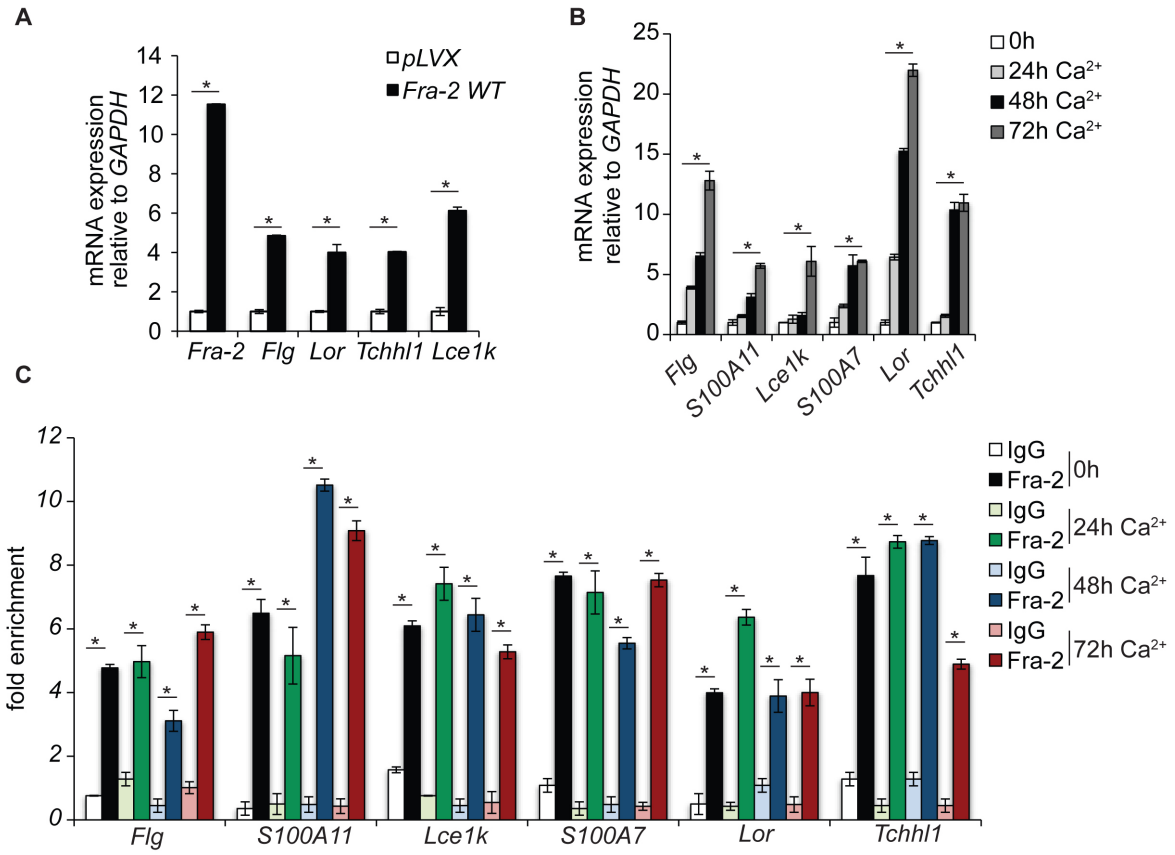


Figure 2. Fra-2 induces EDC gene expression and binds EDC gene promoters. (A) Differential *Fra-2* and EDC gene expression of primary mKCs expressing *Fra-2 WT* compared to empty vector infected cells; n=3**. (B)** EDC mRNA expression analyses during wild-type mKC differentiation (0-72h Ca^{2+}); n=5; **(C)** Chromatin-immunoprecipitation (ChIP) analyses of Fra-2 at EDC promoters during wildtype mKC differentiation (0-72h Ca^{2+}); n=5; * $p<0.05$; bars represent mean ±SD.

1.3. Fra-2 and Ezh2 co-regulate epidermal differentiation genes

Fra-2 ChIP data suggested that transcriptional repressors might co-regulate EDC genes together with AP-1 transcription factors in basal cells. Since PRC2 represses EDC genes (Ezhkova et al., 2009; Mejetta et al., 2011), ChIP studies were performed using antibodies against the PRC2 subunits Ezh2, Suz12 and the repressive histone mark H3K27me3 **(Figure 3)**.

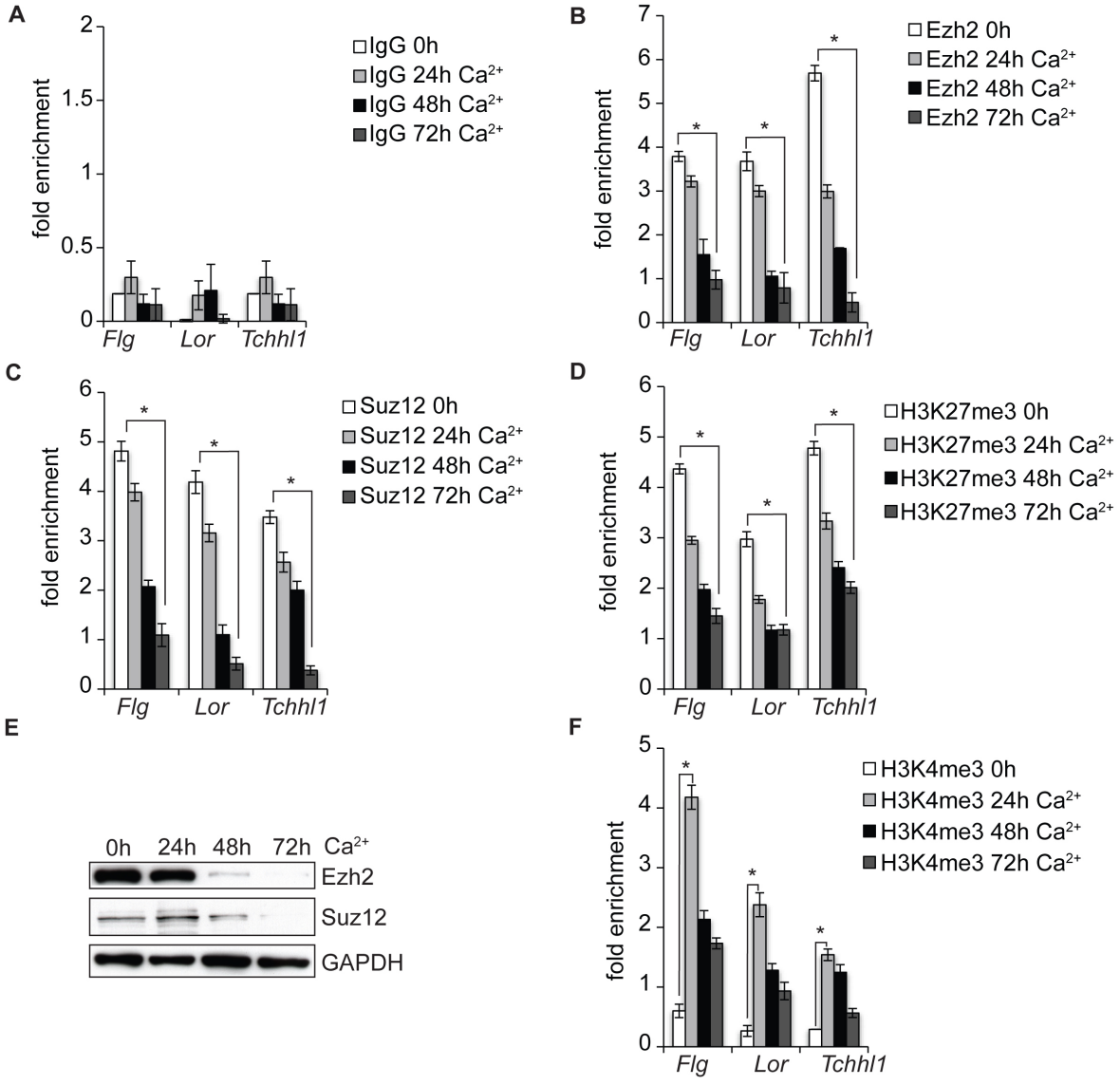


Figure 3. Ezh2/PRC2 co-regulate epidermal differentiation genes. (A-D) ChIP analyses of negative control IgGs **(A)** Ezh2 **(B)**, Suz12 **(C)** and H3K27me3 **(D)** n=3; **(E)** Ezh2 and Suz12 protein expression during *in vitro* mKC differentiation; n=4; **(F)** ChIP analyses of H3K4me3 during mKC differentiation (0-72h Ca^{2+}); n=3; * $p<0.05$, bars represent mean ±SD.

Rabbit IgGs were used as negative control for all ChIP experiments **(Figure 3A)**. The transcription start sites (TSS) of Fra-2-bound EDC genes were found enriched for Ezh2 and Suz12 and positive for H3K27me3 in basal cells. Importantly, the associations of PRC2 subunits as well as H3K27me3 levels were reduced upon Ca^{2+}-induced differentiation **(Figure 3B-D).** These findings are consistent with the drastic reduction of Ezh2 and Suz12 protein levels during mKC differentiation **(Figure 3E)**. In addition to the reduction of the repressive H3K27me3 modification, H3K4me3, a histone modification associated with transcriptional activation, was increased upon mKC differentiation **(Figure 3F)**. These results show that Fra-2/AP-1 and Ezh2/PRC2 co-occupy EDC promoters in basal keratinocytes and that the removal of PRC2 subunits correlates with transcriptional activity.

1.4. Fra-2 and Ezh2 physically interact and Fra-2 is methylated on lysine(s)

Besides modifying histones, Ezh2 methylates non-histone substrates, such as some transcription factors, thereby regulating their transcriptional activity (He et al., 2012; Kim et al., 2013; Lee et al., 2012; Xu et al., 2012). Therefore, we investigated a potential histone methylation-independent role of Ezh2 on Fra-2 during epidermal differentiation employing co-immunoprecipitations (coIP). Ezh2 co-precipitated with Fra-2 and Fra-2 co-precipitated with Ezh2 in protein lysates isolated from basal keratinocytes **(Figure 4A,B)**.

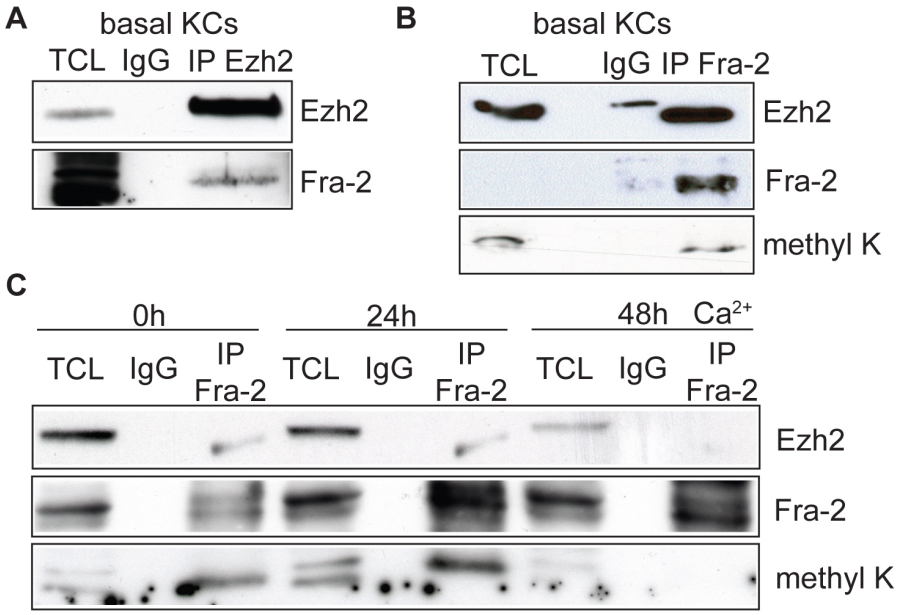


Figure 4. Fra-2 and Ezh2 physically interact. **(A,B)** Co-immunoprecipitation (coIP) of Ezh2 **(A)** and coIP of Fra-2 **(B)** and Western blot (WB) analysis of Ezh2, Fra-2 and methylated lysine (methyl K) in basal mKCs; n=5. **(C)** CoIP of Fra-2 and WB analysis of Ezh2, Fra-2 and methyl K during mKC differentiation (0-48h Ca^{2+}); n=5; TCL=total cell lysate.

Since Ezh2 methylates specific lysine residues of histone proteins and Ezh2 interacts with Fra-2, the methylation status of Fra-2 was investigated. Using immunoprecipitated Fra-2 and an antibody against pan-methyl lysine (methyl K), methylated Fra-2 was detected in basal mKCs **(Figure 4B).** CoIP experiments revealed that the Fra-2-Ezh2 interaction was reduced upon mKC differentiation, consistent with the reduction of Ezh2 protein levels **(Figure 4C)**. In agreement with PRC2 reduction upon differentiation, Fra-2 methylation was absent in differentiated mKCs **(Figure 4C)**. These data demonstrate that Fra-2 and Ezh2 interact and that Fra-2 is methylated on lysine residues in basal cells, correlating with transcriptional inactivity at the EDC locus.

1.5. Ezh2 inhibition reduces Fra-2 methylation and promotes differentiation

To assess a role of Ezh2 in Fra-2 methylation, Ezh2 was pharmacologically targeted using two small molecules, GSK126 and EPZ6438, inhibitors of the methyltransferase domain of Ezh2. The methylation status of Fra-2 protein in basal keratinocytes was assessed by IP for methylated lysine and WB for Fra-2. In both cases, Fra-2 methylation was decreased upon Ezh2 inhibition **(Figure 5A)**.

Pharmacological inhibition of Ezh2 was not sufficient to induce terminal mKC differentiation. However, GSK126 inhibitor-treated cells differentiated prematurely upon Ca^{2+} treatment **(Figure 5B)**. These findings indicate that upon inhibition of Ezh2-mediated methylation, an additional stimulus is required to induce mKC differentiation.

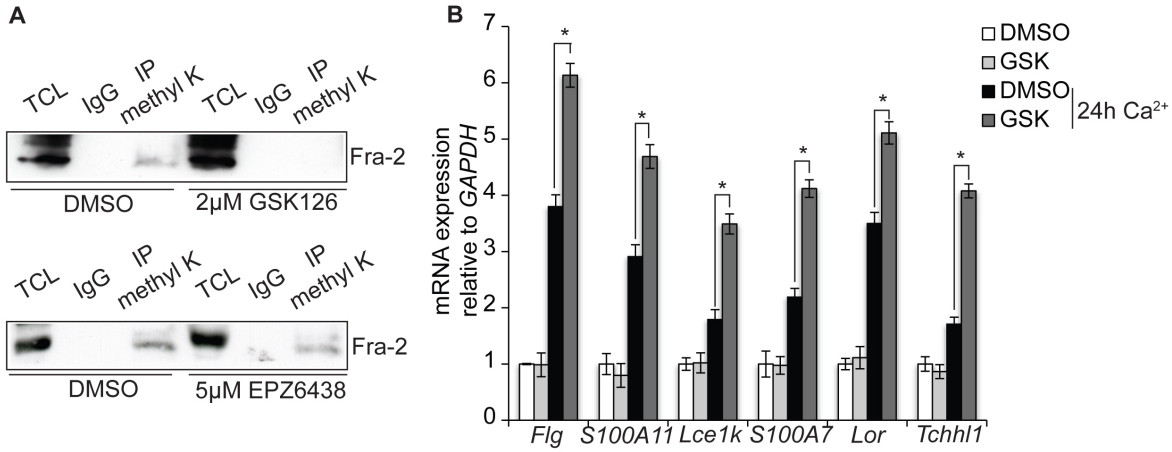


Figure 5. Reduced Fra-2 methylation and increased expression of EDC genes upon inhibition of Ezh2. (A) IP of methyl K and WB of Fra-2 upon Ezh2 inhibition with GSK126 and EPZ6438 in basal mKCs; n=3. **(B)** mRNA expression analyses of EDC genes upon Ezh2 inhibition (GSK126) for 24h with or without Ca^{2+}; n=3; * $p<0.05$, bars represent mean ±SD.

1.6. Fra-2 is methylated on lysine 104 in basal mKCs

To investigate the molecular nature of Fra-2 lysine methylation, endogenous Fra-2 was immunoprecipitated from basal mKC protein extracts followed by mass spectrometry (MS) analyses. In two independent experimental approaches (see Materials and Methods), MS analyses identified mono- and dimethylation of lysine 104 (K104) **(Figure 6A-D)**.

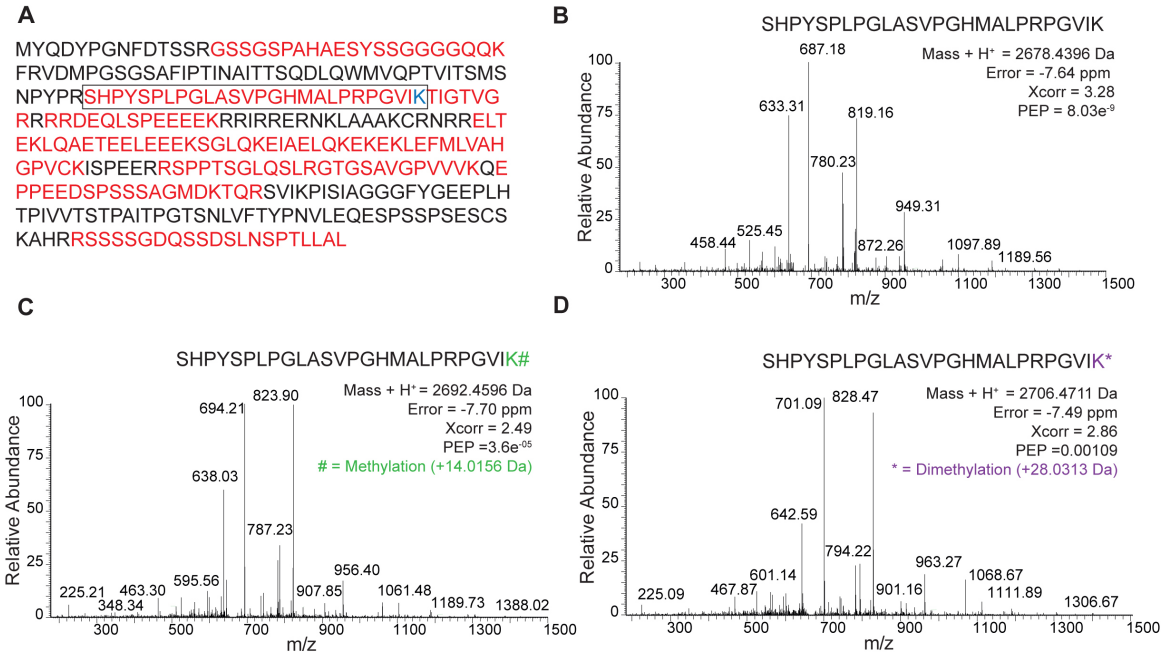


Figure 6. Fra-2 is methylated on lysine 104 (K104) in basal mKCs. (A) Sequence coverage of Fra-2 identified by MS analysis, in which red residues represent the identified peptides. **(B-D)** MS spectra of 89-104 Fra-2 peptide (framed in A) found unmodified **(B)**, mono-methylated on K104 **(C)** and di-methylated on K104 **(D)**. Mass error, identification score and posterior error probability (PEP) are shown for the 3 different Fra-2 89-104 peptides.

1.7. Mimicking Fra-2 lysine methylation decreases Fra-2 transcriptional activity and EDC gene expression in primary mKCs *in vitro*

Site-directed mutagenesis was next performed to substitute K104 with phenylalanine (K104F), mimicking lysine methylation (Huq et al., 2007). Primary *WT* mKCs were infected with lentiviruses expressing *Fra-2 WT*, *Fra-2 K104F* or empty vector (*pLVX*). Efficient Fra-2 expression was verified by WB and qRT-PCR with primers specific for total and ectopically expressed *Fra-2* **(Figure 7A-C)**. Lentivirus-mediated expression of *Fra-2 WT* induced EDC gene expression in primary *WT* mKCs. However, *Fra-2 K104F* was inactive in the same setting as shown in **Figure 7C**. These results suggest that lysine methylation renders Fra-2 transcriptionally inactive.

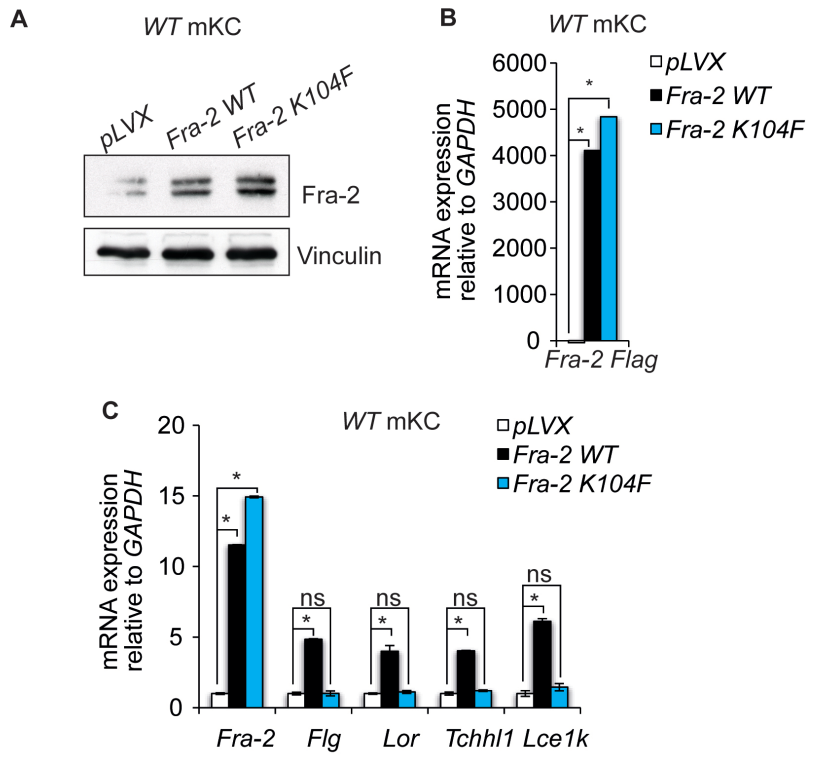


Figure 7. Mimicking Fra-2 K104 methylation reduces the transcriptional activity of Fra-2. (A,B) Fra-2 protein **(A)** and *Fra-2 Flag* gene **(B)** expression of primary mKCs infected with lentiviruses expressing wild-type *Fra-2* (*Fra-2 WT*) or mutant *Fra-2* mimicking lysine methylation (*Fra-2 K104F*) compared to empty vector infected cells (pLVX); n=3. **(C)** Differential *Fra-2* and EDC gene expression of primary mKCs expressing *Fra-2 WT* or *Fra-2 K104F* compared to empty vector infected cells; n=3; * $p<0.05$, bars represent mean ±SD.

1.8. Fra-2 is phosphorylated and stabilized by ERK1/2 upon mKC differentiation

Since Fra-2 mRNA remained unaltered during mKC differentiation, although an increase of total Fra-2 protein was detected, we hypothesized that Fra-2 was stabilized at the protein level by posttranslational modifications. ERK1/2 kinases were shown to stabilize Fra-2 protein by C-terminal serine 320 (S320) and threonine 322 (T322) phosphorylation (Alli et al., 2013). We therefore investigated ERK1/2 activity during mKC differentiation. ERK1/2 phosphorylation was detected in suprabasal keratinocytes of developing embryos at embryonic day 17.5 (E17.5) **(Figure 8A)** and upon Ca^{2+}-induced differentiation *in vitro* **(Figure 8B)**. To investigate the C-terminal Fra-2 serine phosphorylation, a phospho-Fra-1 antibody reacting with a conserved phospho peptide on Fra-1 and Fra-2 (Ser267 and Ser320, respectively) was used on immunoprecipitated Fra-2 at different time points of mKC differentiation. C-terminal Fra-2 phosphorylation was increased upon Ca^{2+}-induced differentiation correlating with increased ERK1/2 phosphorylation **(Figure 8B,C)**.

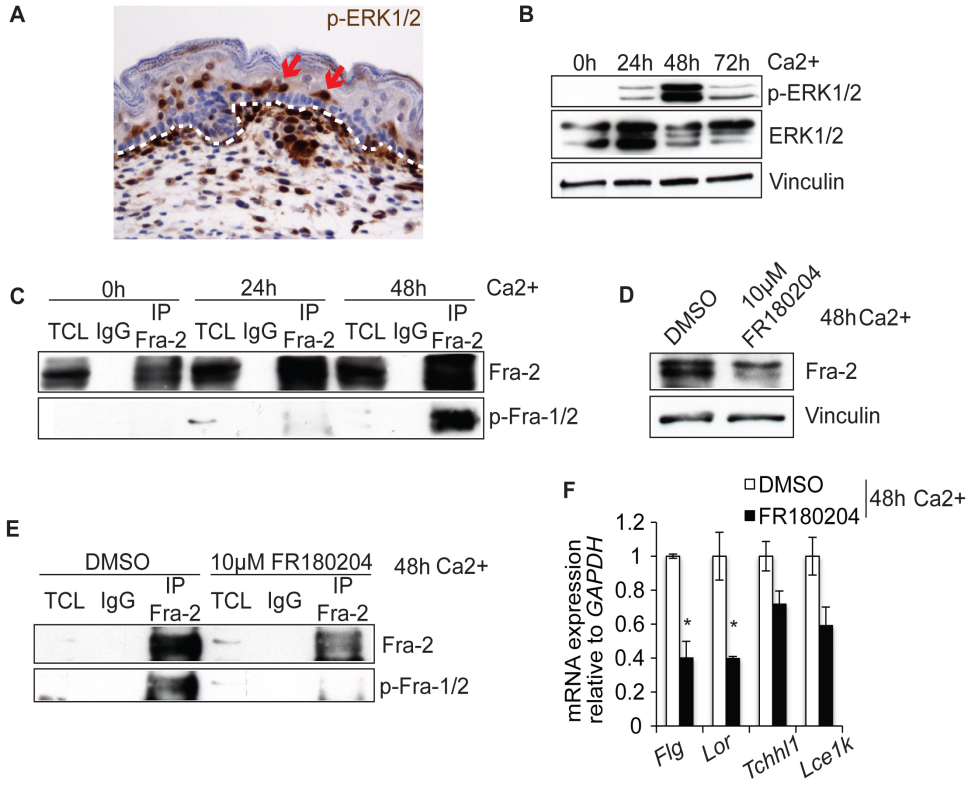


Figure 8. Fra-2 is phosphorylated and stabilized by ERK1/2 upon mKC differentiation (A) p-ERK1/2 IHC in skin of wildtype embryos at E17.5; arrows point towards suprabasal expression, dashed white line indicates basement membrane; n=3. **(B)** ERK1/2 activation shown by p-ERK1/2 WB during *in vitro* mKC differentiation (0-72h Ca^{2+}); n=3. **(C)** IP of Fra-2 during mKC differentiation (0-48h Ca^{2+}) and WB analyses of Fra-2 and p-Fra-1/2; n=3. **(D)** WB analysis of Fra-2 upon Ca^{2+} treatment (48h) and ERK1/2 inhibition with FR180204; n=3. **(E)** IP of Fra-2 upon Ca^{2+} treatment (48h) and ERK1/2 inhibition with FR180204 and WB of Fra-2 and p-Fra-1/2; n=3. **(F)** mRNA expression analyses of EDC genes upon Ca^{2+} treatment (48h) and ERK1/2 inhibition with FR180204; n=3, * $p<0.05$, bars represent mean ±SD.

A small molecule inhibitor of ERK1/2 (FR180204) was next used. ERK1/2 inhibitor treatment of mKCs led to reduced Fra-2 protein levels, reduced Fra-2 phosphorylation as well as reduced EDC gene expression upon Ca^{2+} treatment **(Figure 8D-F)**. Altogether, these data suggest that Fra-2 is stabilized by C-terminal phosphorylation during mKC differentiation and that ERK1/2 activation correlates with Fra-2 phosphorylation.

1.9. C-terminal phosphorylation increases the transcriptional activity of Fra-2 and EDC gene expression in primary mKCs *in vitro*

We next functionally validated the role of C-terminal Fra-2 phosphorylation on EDC gene expression and mutated S320 and T322 to alanine (S320A/T322A), which cannot be phosphorylated. Primary mKCs were infected with lentiviruses expressing *Fra-2 WT*, *Fra-2 S320A/T322A* or empty vector. Efficient Fra-2 expression was verified by WB and qRT-PCR with primers specific for total and ectopically expressed *Fra-2* **(Figure 9A-C)**. While lentivirus-mediated expression of *Fra-2 WT* induced EDC gene expression, expression of *Fra-2 S320A/T322A* did not **(Figure 9C)**. These findings indicate that the ability of Fra-2 to induce EDC gene expression is reduced upon mutation of C-terminal phospho-acceptor sites.

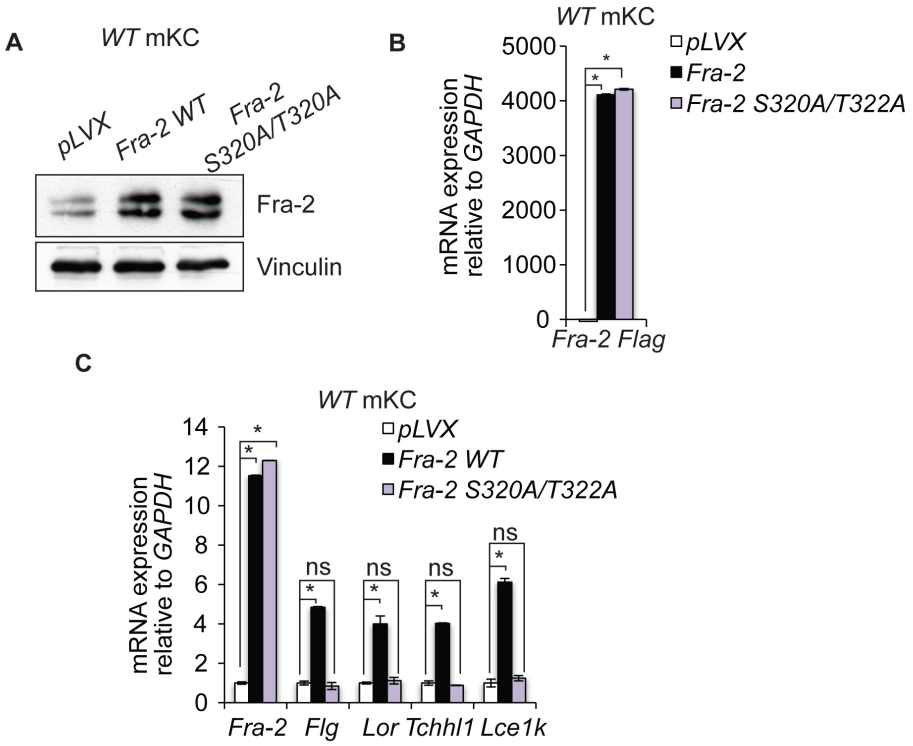


Figure 9. Mutation of Fra-2 C-terminal phospho-acceptor sites reduces the transcriptional activity of Fra-2. (A,B) Fra-2 protein **(A)** and *Fra-2 Flag* gene **(B)** expression of primary mKCs infected with lentiviruses expressing *Fra-2 WT* or mutant Fra-2 deficient for C-terminal phospho-acceptor sites (*Fra-2 S320A/T322A*) compared to empty vector infected cells; n=3. **(C)** Differential *Fra-2* and EDC gene expression of primary mKCs expressing *Fra-2 WT* or *Fra-2 S320A/T322A* compared to empty vector infected cells; n=3; * $p<0.05$, bars represent mean ±SD.

In conclusion, these results show an inverse kinetic of lysine methylation - loss of interaction with Ezh2 - and ERK1/2-mediated serine phosphorylation of Fra-2. At EDC promoters, methylated but not phosphorylated Fra-2 correlates with EDC gene inactivation, while loss of Fra-2 methylation, ERK1/2 activation and Fra-2 phosphorylation correlates with active Fra-2-dependent gene expression **(Figure 10)**.

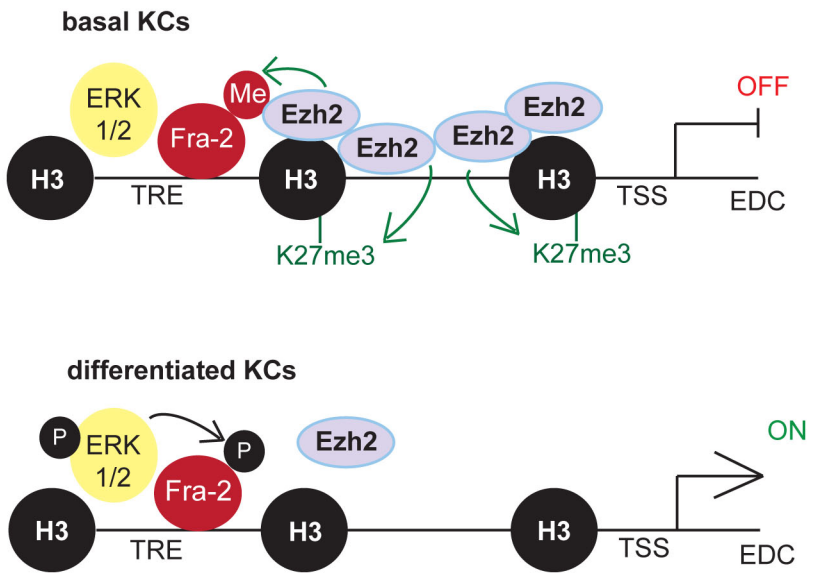


Figure 10. Schematic model of EDC gene regulation by Fra-2, Ezh2 and ERK1/2: in basal cells, methylated Fra-2 (Fra-2 Me) bound at TPA-responsive elements (TREs) of EDC gene promoters and interacting with Ezh2 prevents EDC gene expression; upon mKC differentiation, Fra-2 becomes phosphorylated (Fra-2 P) by ERK1/2 and loses its methylation, Ezh2 protein levels and histone 3 lysine 27 (H3K27) methylation at transcription start sites (TSS) of EDC promoters are reduced, which together induce EDC gene expression.

1.10. Fra-2 induces terminal epidermal differentiation *in vivo*

Given our biochemistry data from *in vitro* experiments, we decided to investigate the function of Fra-2 in regulating epidermal differentiation *in vivo*. For this purpose, genetically engineered mouse models (GEMMs) with epithelial-specific ectopic expression, as well as specific deletion of *Fra-2* were generated. The expression of genes located within the EDC such as *Flg, Lor, Tchhl1* and *Lce1k* was analyzed in these model systems.

Upon ectopic expression of *Fra-2* in K5-expressing epithelia (*Fra-2*$^{Ep\text{-}tetOFF}$), a significant increase of EDC gene transcripts was detected in epidermal samples of embryos at E17.5 (**Figure 11A**). This finding recapitulated our results from *in vitro* experiments with lentivirus-mediated over-expression of Fra-2.

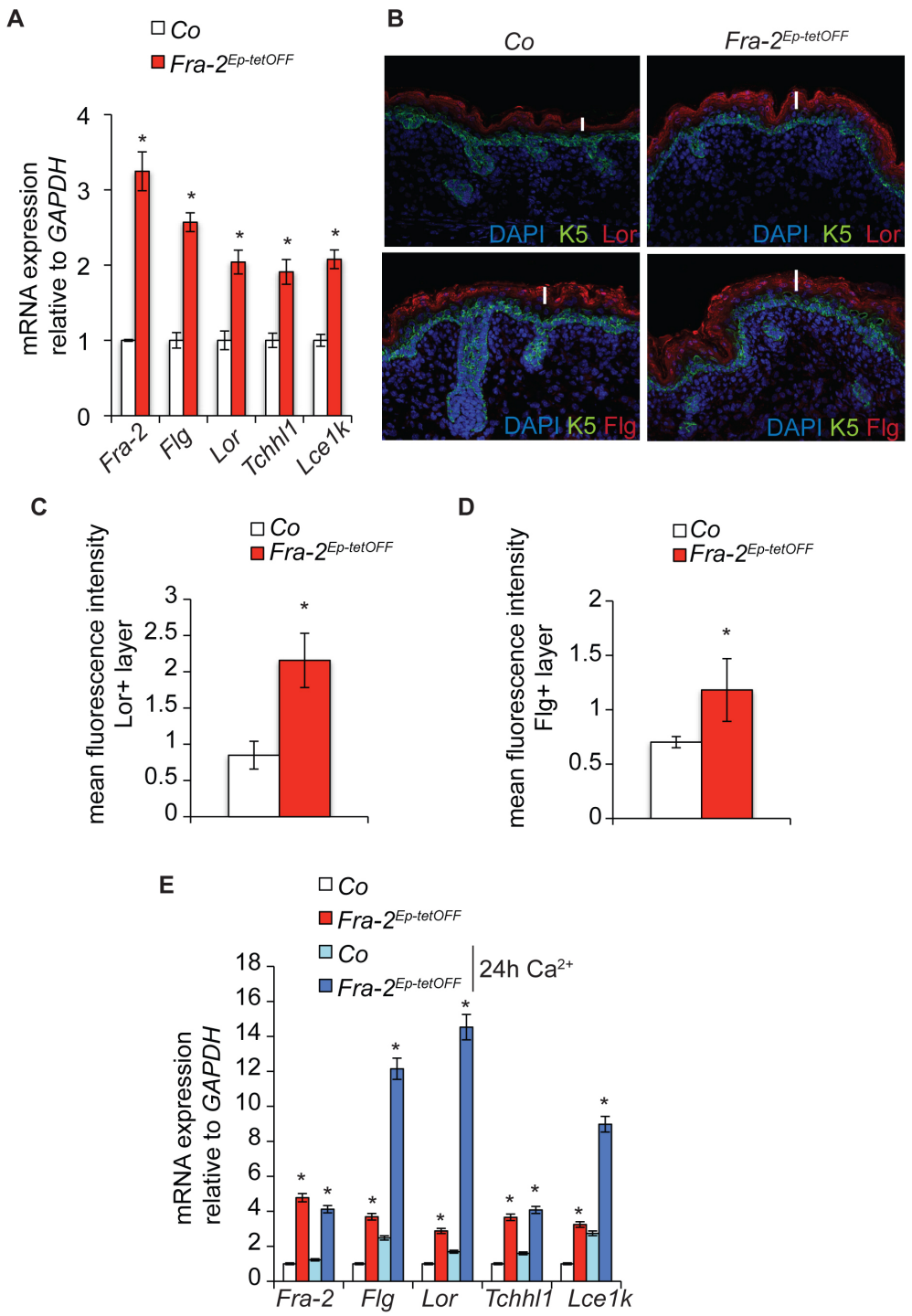


Figure 11. Fra-2 induces terminal epidermal differentiation. (A) Differential EDC gene and *Fra-2* expression in epidermal samples of mutants ectopically expressing Fra-2 (*Fra-2*$^{Ep\text{-}tetOFF}$) compared to control littermates at E17.5; n=6/genotype. **(B)** Basal cell marker expression (K5, green) and late differentiation marker expression (Lor, red, upper panel; Flg, red, lower panel) in *Co* and *Fra-2*$^{Ep\text{-}tetOFF}$ skin at E17.5; n=6/genotype; 40x magnification; white bars indicate thickness of differentiated epidermal layers. **(C,D)** Quantification of Figure 11B; mean Lor **(C)** and Flg **(D)** fluorescence intensity/40x field; n=10 fields/genotype. **(E)** Differential EDC gene and *Fra-2* expression in primary *Fra-2*$^{Ep\text{-}tetOFF}$ mKCs compared to control mKCs under basal conditions and upon Ca^{2+} treatment for 24h *in vitro*; n=3; * p<0.05; bars represent mean ±SD.

The increase of EDC gene expression was accompanied by an increase of terminally differentiated epidermal layers in *Fra-2*$^{Ep\text{-}tetOFF}$ mutants (**Figure 11B, quantified in Figure 11C,D**).

The consequences of increased *Fra-2* expression on mKC homeostasis under stress conditions were next investigated. Primary mKCs isolated from *Fra-2*$^{Ep\text{-}tetOFF}$ mutants underwent precocious differentiation in culture characterized by increased expression of terminal differentiation genes at basal conditions, which was more pronounced upon Ca^{2+} treatment **(Figure 11E),** again confirming our previous *in vitro* results.

Collectively, these findings suggest that Fra-2 induces terminal mKC differentiation *in vivo* and in primary mKCs *in vitro*, while the proliferation of basal mKCs in *vivo*, assessed by Ki67 staining, remained unaffected **(Figure 12A,B)**.

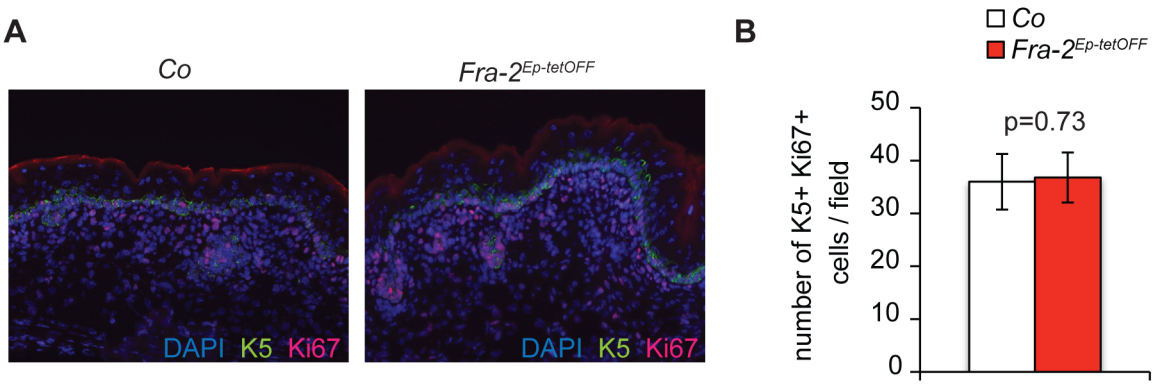


Figure 12. Ectopic *Fra-2* expression does not affect mKC proliferation. (A) Basal cell marker expression (K5, green) and proliferation marker expression (Ki67, red) in *Co* and *Fra-2*$^{Ep\text{-}tetOFF}$ embryos at E17.5; n=5/genotype; 20x magnification. **(B)** Quantification of Figure 12A, K5+/Ki67+ cells/20x field; n=10 fields/genotype; bars represent mean ±SD.

To validate these findings in a more disease-relevant setting, *Fra-2* was ectopically expressed in papilloma-prone *K5-SOS-F* transgenic mice (*Fra-2*$^{Ep\text{-}tetOFF}$ *SOS*+). Interestingly, skin papillomas of poorly differentiated *SOS*+ mice show high levels of Ezh2 and express Fra-2 **(Figure 13)**.

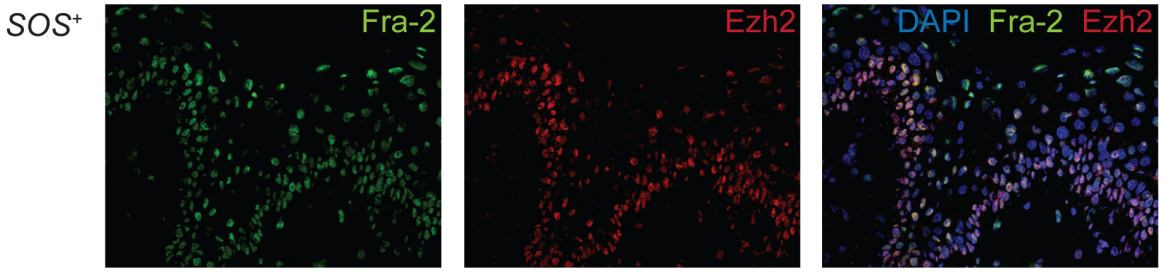


Figure 13. *SOS*+ papillomas express Ezh2 and Fra-2. IF analysis of Fra-2 (green) and Ezh2 (red) in *SOS*+ mice at 5 weeks of age; n=5; nuclei are stained with dapi (blue).

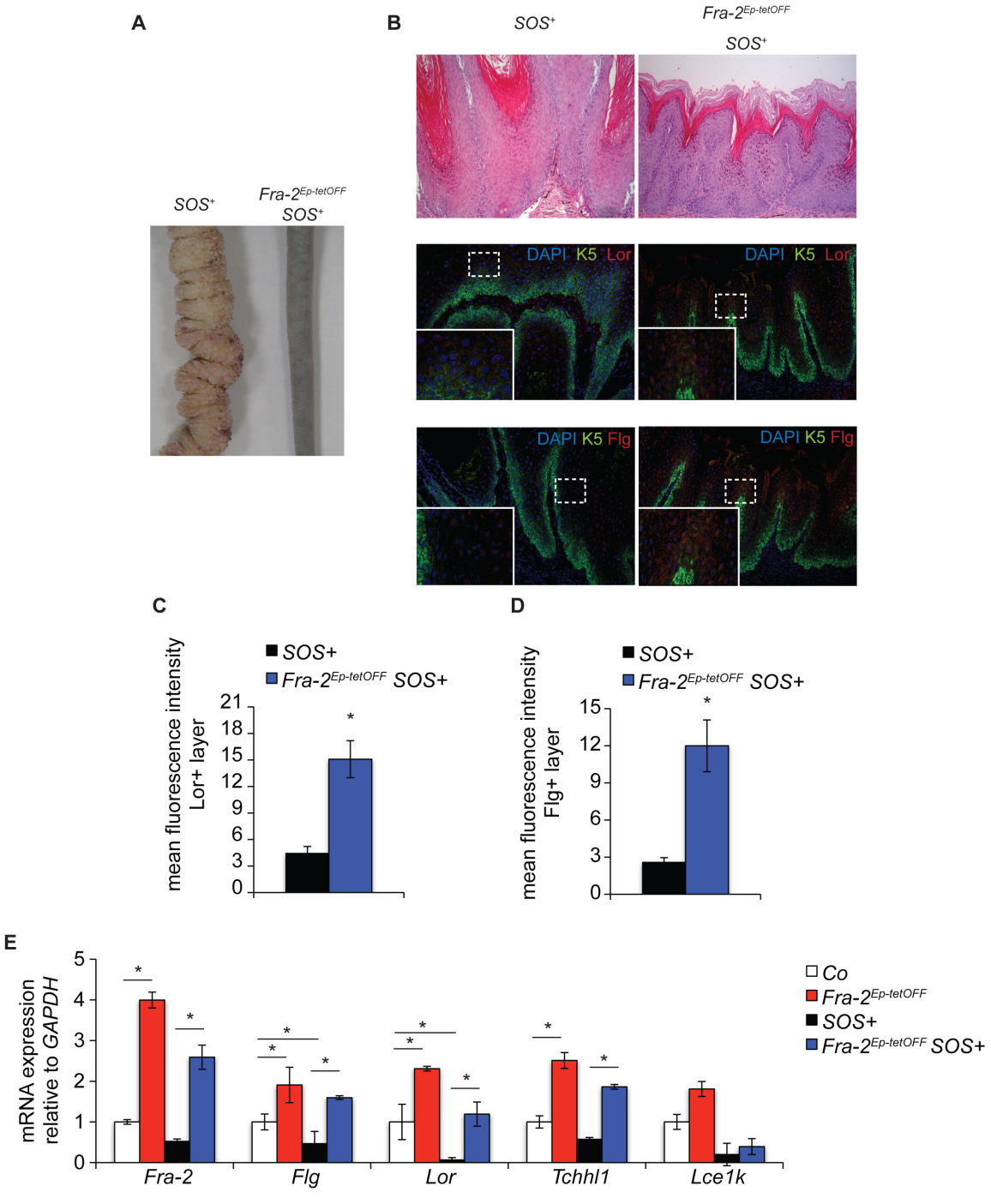


Figure 14. Fra-2 suppresses skin papilloma growth in *SOS+* mice by inducing differentiation. **(A)** Macroscopic appearance of *SOS+* and *Fra-2*$^{Ep\text{-}tetOFF}$ *SOS+* papillomas at 5 weeks of age; n=6/genotype. **(B)** Analyses of *SOS+* and *Fra-2*$^{Ep\text{-}tetOFF}$ *SOS+* papillomas. H&E (upper panel), basal cell marker expression (K5, green) and late differentiation marker expression (Lor, red, middle panel; Flg, red, lower panel) in *SOS+* and *Fra-2*$^{Ep\text{-}tetOFF}$ *SOS+* papillomas at 5 weeks of age; 20x magnification, insert depicts 40x magnification; n=6/genotype. **(C,D)** Quantification of Figure 14B: mean Lor **(C)** and Flg **(D)** fluorescence intensity/20x field; n=10 fields/genotype. **(E)** Differential EDC gene and *Fra-2* expression in *Co*, *Fra-2*$^{Ep\text{-}tetOFF}$, *SOS+* and *Fra-2*$^{Ep\text{-}tetOFF}$ *SOS+* mice at 5 weeks of age; n=4/genotype; * $p<0.05$; bars represent mean ±SD.

Importantly, ectopic expression of Fra-2 in this background strongly suppressed skin papilloma growth with significantly smaller lesions in *Fra-2*$^{Ep\text{-}tetOFF}$ *SOS*+ mice **(Figure 14A, B H&E upper panel)**. Importantly, increased expression of EDC genes was detected by IF **(Figure 14B, middle and lower panel, quantified in Figure 14C,D)** and mRNA analyses **(Figure 14E)**, while no changes in proliferation were observed **(Figure 15A,B)**.

These results demonstrate that epidermal Fra-2 can induce EDC gene expression under stress conditions and functions as a suppressor of papilloma growth due to the induction of epidermal differentiation.

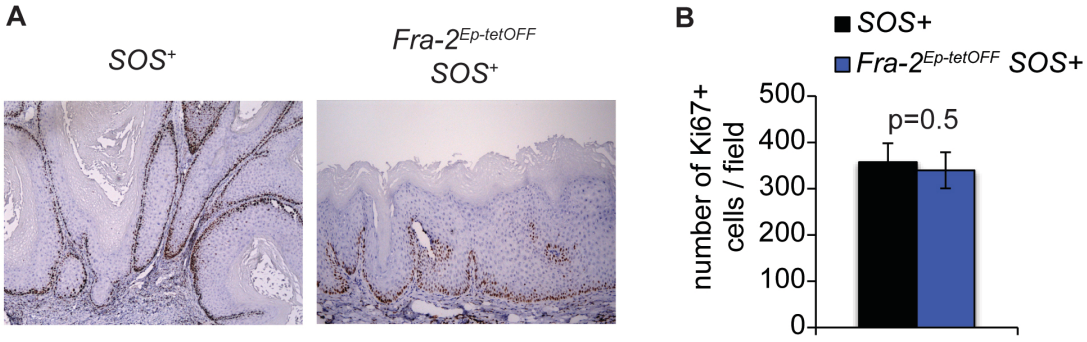


Figure 15. Ectopic *Fra-2* expression does not affect mKC proliferation in *SOS*+ mice. (A) Proliferation marker expression (Ki67, brown) in *SOS*+ and *Fra-2*$^{Ep\text{-}tetOFF}$ *SOS*+ mice at 5 weeks of age; n=5/genotype. **(B)** Quantification of Figure 15A, Ki67+ cells/field; n=5 fields/genotype; bars represent mean ±SD.

1.11. Fra-2 is necessary for proper epidermal differentiation

GEMMs with *Fra-2* deletion in the differentiated compartment of the epidermis (suprabasal deletion with FoxN1-Cre; *Fra-2*$^{\Delta sb}$) and with *Fra-2* deletion in the entire epidermis (basal deletion with K5-Cre; *Fra-2*$^{\Delta ep}$) were generated **(Figure 16A)**.

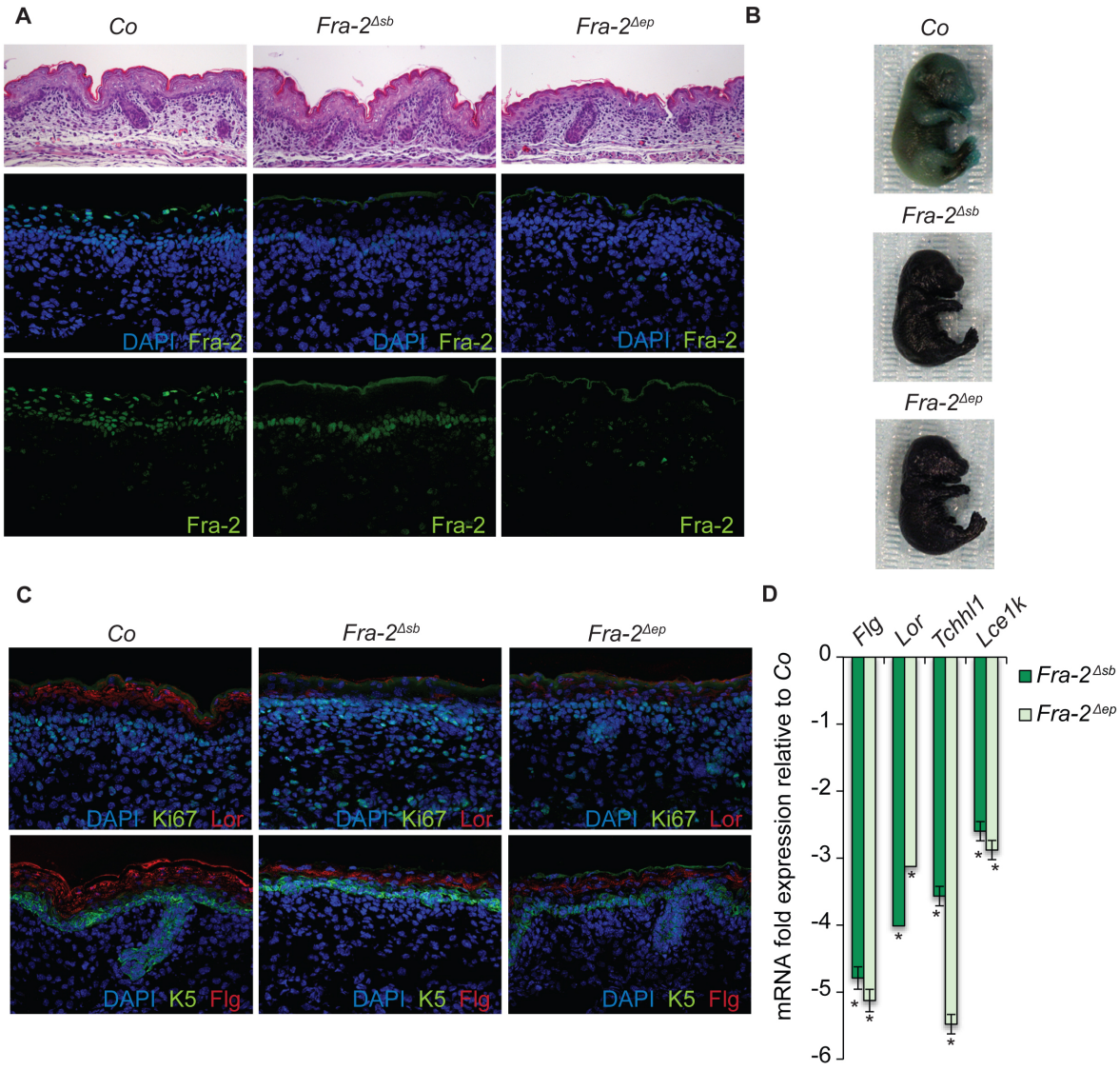


Figure 16. Fra-2/AP-1 is necessary for proper epidermal differentiation. (A) H&E and Fra-2 IF of *Co* (*Fra-2*$^{f/f}$), *Fra-2*$^{\Delta sb}$ (*Fra-2*$^{f/f}$*; FoxN1 Cre*$^{+/T}$) and *Fra-2*$^{\Delta ep}$ (*Fra-2*$^{f/f}$*; K5 Cre*$^{+/T}$) embryos at E17.5; n=6/genotype; 40x magnification. **(B)** Toluidine blue dye penetration assay in *Co*, *Fra-2*$^{\Delta sb}$ and *Fra-2*$^{\Delta ep}$ embryos at E17.5; n=6/genotype. **(C)** Proliferation/basal cell marker expression (Ki67, green, upper panel; K5, green, lower panel) and late differentiation marker expression (Lor, red, upper panel; Flg, red, lower panel) in *Co*, *Fra-2*$^{\Delta sb}$ and *Fra-2*$^{\Delta ep}$ embryos at E17.5; n=6/genotype; 40x magnification. **(D)** Differential EDC gene expression in *Fra-2*$^{\Delta sb}$ and *Fra-2*$^{\Delta ep}$ embryos compared to control littermates at E17.5; n=4/genotype; * $p<0.05$, bars represent mean ±SD.

While macroscopically indistinguishable from control littermates at E17.5, Toluidine blue dye penetration assays, performed to assess the functionality of the outside-in-barrier, revealed a persistent defect in skin barrier formation in both *Fra-2*$^{\Delta sb}$ and *Fra-2*$^{\Delta ep}$ mutants **(Figure 16B)**. Importantly, we measured a significant reduction in the expression of EDC genes in these mutants **(Figure 16C, D)**. Thus, the observed skin barrier defect is likely a consequence of incomplete epidermal differentiation. Hence, Fra-2 appears to be necessary for correct terminal epidermal differentiation *in vivo*. Notably, the proliferation status of basal mKCs remained unaffected at E17.5 in *Fra-2*$^{\Delta sb}$ and *Fra-2*$^{\Delta ep}$ mutants **(Figure 17A, B)**. Although Fra-2 is also expressed in basal cells, the phenotype of *Fra-2*$^{\Delta sb}$ mutants suggests that Fra-2 mainly functions in the differentiated compartment of the epidermis.

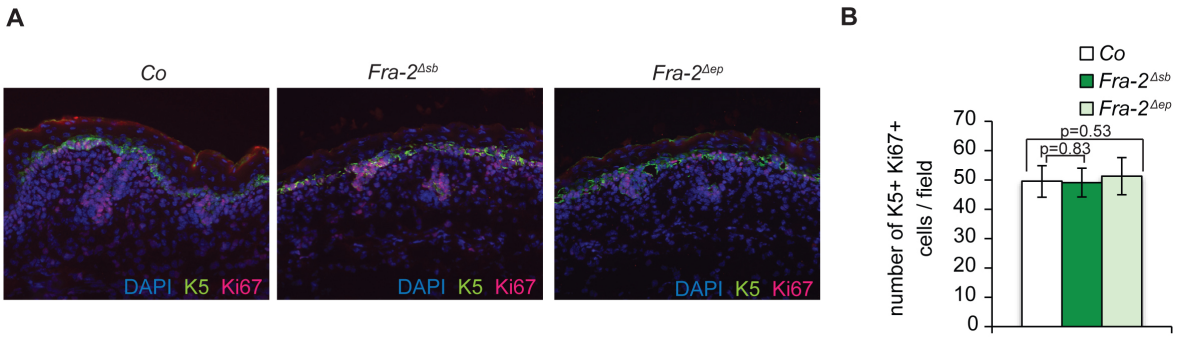


Figure 17. Loss of epidermal Fra-2 does not affect mKC proliferation. (A) Basal cell marker expression (K5, green) and proliferation marker expression (Ki67, red) in *Co*, *Fra-2*$^{\Delta sb}$ and *Fra-2*$^{\Delta ep}$ embryos at E17.5; n=5/genotype; 20x magnification. **(B)** Quantification of Figure 17A, K5+/Ki67+ cells/20x field; n=10 fields/genotype; bars represent mean ±SD.

1.12. Cell autonomous differentiation defect in primary Fra-2-deficient mKCs

The expression of EDC genes was next investigated in primary Fra-2-deficient mKCs *in vitro*. For this purpose, primary mKCs carrying Fra-2 loxed alleles (*Fra-2*$^{f/f}$) were infected with adeno-*Cre* (*AdCre*) viruses for *in vitro* Cre-mediated *Fra-2* inactivation and adeno-*GFP* (*AdGFP*) control viruses. Cre-mediated recombination was assessed by measuring non-functional *Fra-2Δ-GFP* fusion gene expression, which is produced upon excision of exon 3 of *Fra-2* and fusion of exon 2 with a *GFP* reporter (Eferl et al., 2007) **(Figure 18A).** Notably, upon deletion of *Fra-2 in vitro*, the EDC genes *Flg*, *Lor* and *Lce1k* were significantly reduced in primary mKCs **(Figure 18B).** These results further confirm that Fra-2 is necessary for the correct expression of these EDC genes.

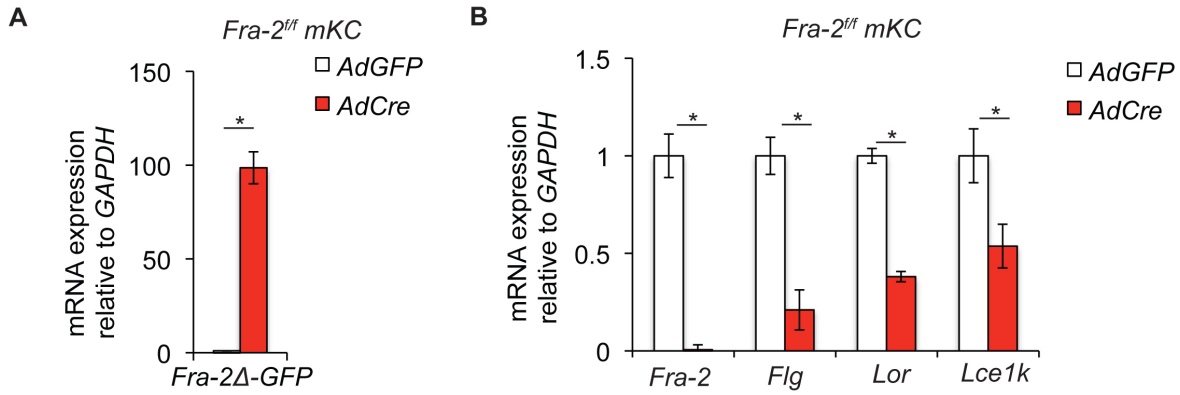


Figure 18. Reduced EDC expression in primary Fra-2-deficient mKCs. (A) *Fra-2Δ-GFP* mRNA expression of primary mKCs carrying Fra-2 loxed alleles (*Fra-2$^{f/f}$*) infected with adeno-*GFP* (*AdGFP*) or adeno-*Cre* (*AdCre*) viruses. **(B)** Differential *Fra-2* and EDC gene expression of primary *Fra-2$^{f/f}$* mKCs infected with *AdGFP* or *AdCre;* n=3; * p<0.05, bars represent mean ±SD.

To validate our previous findings, *Fra-2 WT* and *Fra-2 K104F* as well as *Fra-2 S320A/T322A* mutants were expressed lentivirally as described above in primary Fra-2-deficient mKCs and the expression of EDC genes was analyzed.

1.13. Expression of a *Fra-2 K104F* mutant does not rescue reduced EDC gene expression in Fra-2-deficient mKCs

First, the effect of *Fra-2 K104F* expression on EDC gene expression in Fra-2-deficient mKCs compared to *Fra-2 WT* expression was evaluated. Cre-mediated recombination was assessed by measuring non-functional *Fra-2Δ-GFP* fusion gene expression **(Figures 19A).** Efficient *Fra-2 WT* and *Fra-2 K104F* expression was assessed by exogenous *Fra-2 Flag* and total *Fra-2* expression in these cells **(Figures 19B,C)**. While lentiviral expression of *Fra-2 WT* restored the differentiation defect of primary Fra-2-deficient mKCs, the expression of the *Fra-2 K104F* mutant did not **(Figure 19C)**. These results further confirm that lysine methylation of K104 decreases the transcriptional activity of Fra-2 on EDC genes in primary mKCs and thus the expression of a lysine-methylation-mimicking mutant does not rescue cell autonomous differentiation defects in Fra-2-deficient mKCs.

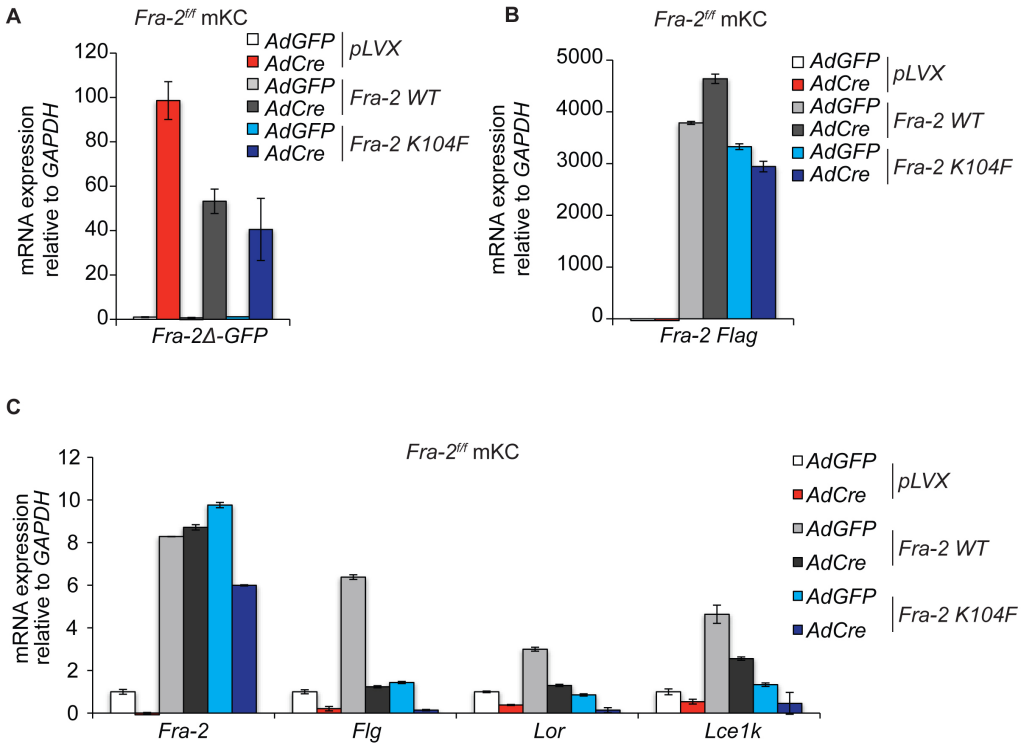


Figure 19. Expression of a *Fra-2 K104F* mutant does not rescue reduced EDC gene expression in Fra-2-deficient mKCs. (A) *Fra-2Δ-GFP* mRNA expression of primary *Fra-2*$^{f/f}$ mKCs infected with *AdGFP* or *AdCre* viruses, expressing *Fra-2 WT* or *Fra-2 K104F* mutant compared to empty vector infected cells; n=3. **(B)** Exogenous *Fra-2-Flag* mRNA expression of primary *Fra-2*$^{f/f}$ mKCs infected with *AdGFP* or *AdCre*, expressing *Fra-2 WT* or *Fra-2 K104F* mutant compared to empty vector infected cells; n=3. **(C)** Differential *Fra-2* and EDC gene expression of primary *Fra-2*$^{f/f}$ mKCs infected with *AdGFP* or *AdCre*, expressing *Fra-2 WT* or *Fra-2 K104F* compared to empty vector infected cells; n=3; bars represent mean ±SD.

1.14. Expression of a *Fra-2 S320A/T322A* mutant does not rescue reduced EDC gene expression in Fra-2-deficient mKCs

Next, the effect of *Fra-2 S320A/T322A* expression on EDC gene expression in Fra-2-deficient mKCs compared to *Fra-2 WT* expression was evaluated. Cre-mediated recombination was assessed by measuring non-functional *Fra-2Δ-GFP* fusion gene expression **(Figures 20A).** Efficient *Fra-2 WT* and *Fra-2 S320A/T322A* expression was assessed by exogenous *Fra-2 Flag* and total *Fra-2* expression in these cells **(Figures 20B,C)**. While lentiviral expression of *Fra-2 WT* restored the differentiation defect of primary Fra-2-deficient mKCs, the expression of the *Fra-2 S320A/T322A* mutant did not **(Figure 20C)**. These findings confirm that the ability of Fra-2 to induce EDC gene expression is reduced upon mutation of C-terminal phospho-acceptor sites and thus the expression of a phospho-deficient mutant does not rescue cell autonomous differentiation defects in Fra-2-deficient mKCs.

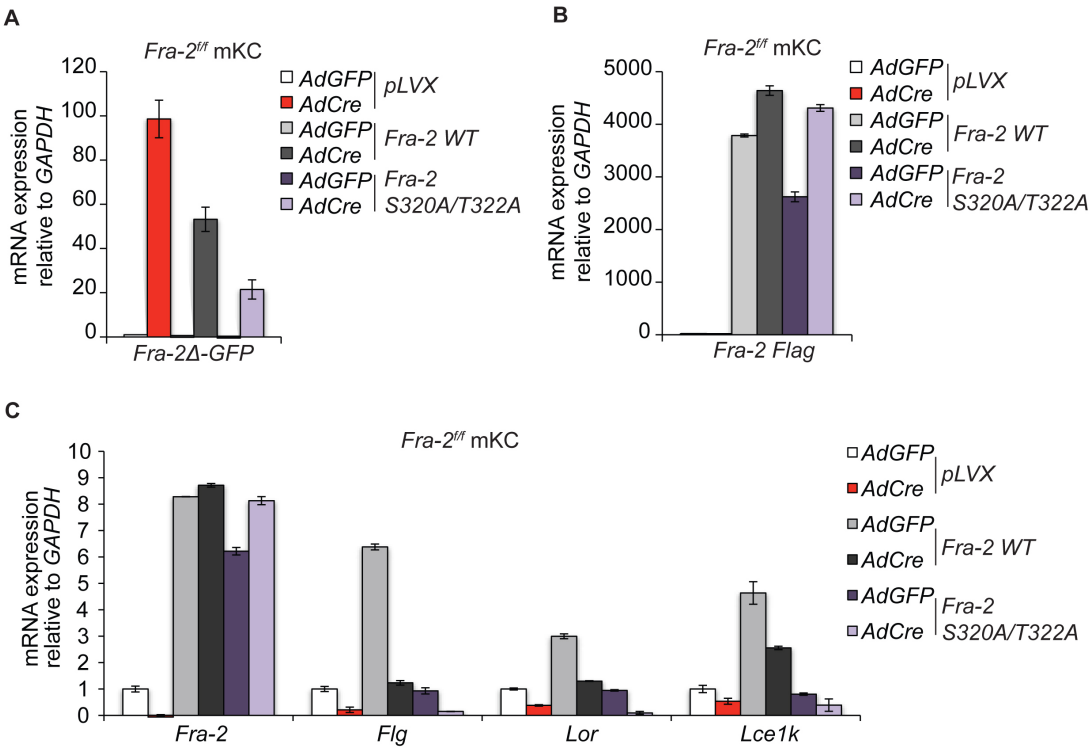


Figure 20. Expression of a *Fra-2 S320A/T322A* mutant does not rescue reduced EDC expression in Fra-2-deficient mKCs. (A) *Fra-2Δ-GFP* mRNA expression of primary *Fra-2$^{f/f}$* mKCs infected with *AdGFP* or *AdCre* viruses, expressing *Fra-2 WT* or *Fra-2 S320A/T322A* mutant compared to empty vector infected cells; n=3. **(B)** Exogenous *Fra-2-Flag* mRNA expression of primary *Fra-2$^{f/f}$* mKCs infected with *AdGFP* or *AdCre*, expressing *Fra-2 WT* or *Fra-2 S320A/T322A* mutant compared to empty vector infected cells; n=3. **(C)** Differential *Fra-2* and EDC gene expression of primary *Fra-2$^{f/f}$* mKCs infected with *AdGFP* or *AdCre*, expressing *Fra-2 WT* or *Fra-2 S320A/T322A* compared to empty vector infected cells; n=3; bars represent mean ±SD.

2. Loss of Fra-2 expression results in skin inflammation

2.1. Epidermal hyperplasia and inflammation upon loss of epidermal Fra-2

Altered epidermal differentiation characterizes a multitude of skin diseases. I next investigated how the differentiation defects in Fra-2 loss-of-function (LOF) mutants affect skin homeostasis. Efficient deletion of *Fra-2* in differentiated mKCs (*Fra-2*$^{\Delta sb}$) or all mKCs (*Fra-2*$^{\Delta ep}$) was evaluated by Fra-2 IHC **(Figure 21A)**. Interestingly, both *Fra-2*$^{\Delta sb}$ and *Fra-2*$^{\Delta ep}$ mutants displayed striking skin abnormalities at postnatal day 6 (P6) characterized by a thinner dermal layer marked by aberrant hair follicle development **(Figure 21B; quantified in Figure 21C)** and epidermal hyperplasia with significantly increased epidermal thickness **(Figure 21D; quantified in Figure 21E)**.

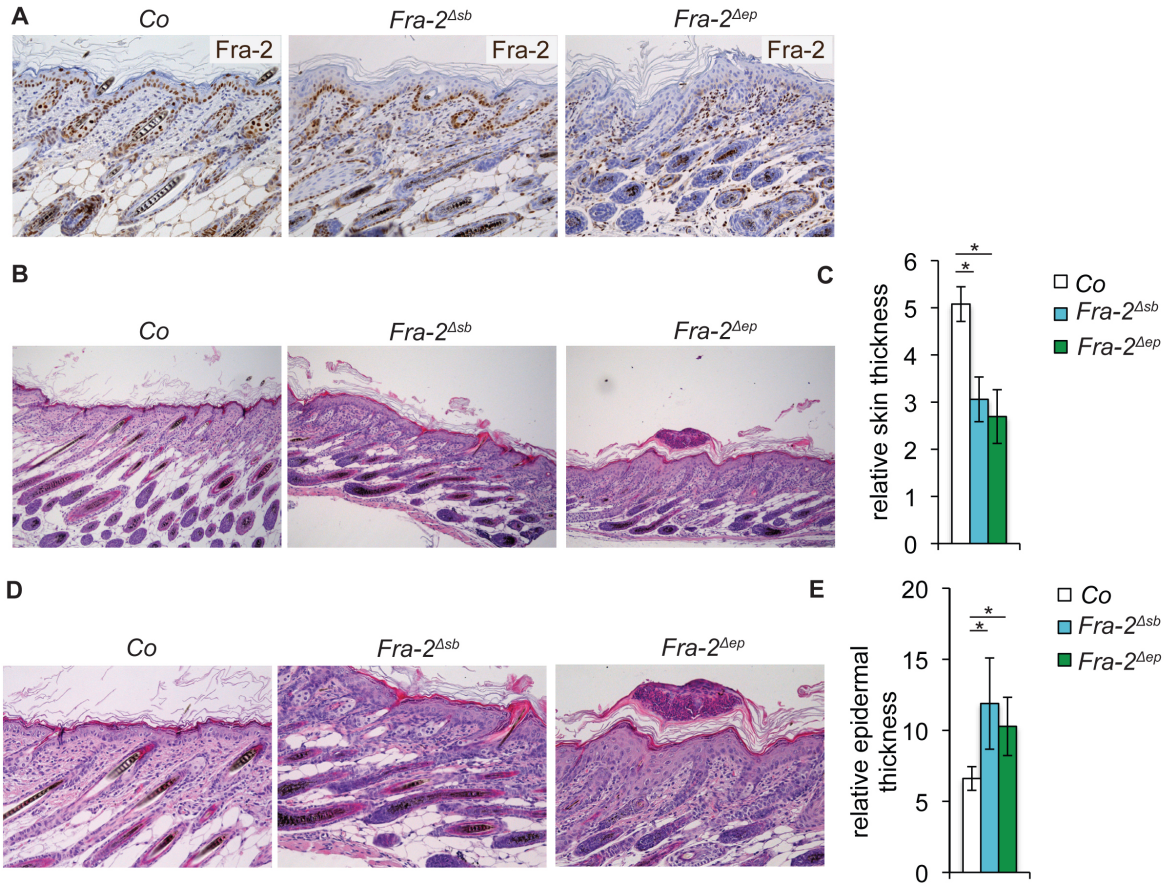


Figure 21. Loss of epidermal Fra-2 results in skin alterations. (A) Fra-2 IHC, H&E analyses to evaluate total skin thickness **(B,C)** and epidermal thickness **(D,E)** of *Co*, *Fra-2*$^{\Delta sb}$ and *Fra-2*$^{\Delta ep}$ pups at postnatal day (P6); n=8/genotype; * p<0.05, bars represent mean ±SD.

While no difference of mKC proliferation was observed during embryonic development, IHC analyses showed that the epidermal phenotype of Fra-2 LOF mutants at P6 was marked by keratinocyte hyperproliferation, depicted by increased numbers of Ki67 positive keratinocytes **(Figure 22A; quantified in Figure 22B)**. Further, increased numbers of CD45 positive inflammatory infiltrates predominantly in the upper dermis were found in Fra-2 LOF mutants **(Figure 22C; quantified in Figure 22D)**.

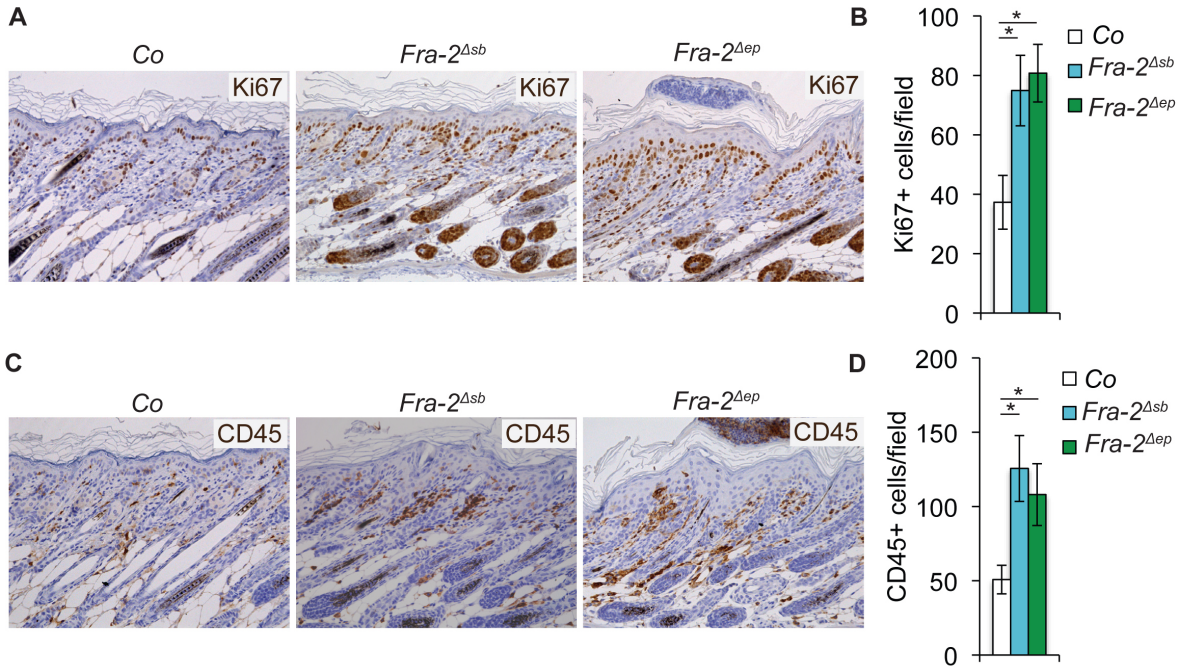


Figure 22. Loss of epidermal Fra-2 results in keratinocyte hyperproliferation and skin inflammation. (A) Ki67 IHC, **(B)** quantification of Ki67 positive cells, **(C)** CD45 IHC, **(D)** quantification of CD45 positive cells of *Co*, *Fra-2*$^{\Delta sb}$ and *Fra-2*$^{\Delta ep}$ pups at P6; n=6/genotype; * p<0.05, bars represent mean ±SD.

Besides the inflammatory skin phenotype, Fra-2 LOF mutants were severely growth retarded and cachectic at P6, presented less than 50% of the body weight of littermate controls **(Figure 23A)** and died within the first two weeks after birth with complete penetrance. Notably, upon loss of epidermal Fra-2, a systemic inflammatory response characterized by increased levels of the cytokines TNFα, IL-6, IL-17A, G-CSF, IL-1α and TSLP in sera was observed **(Figure 23B)**. Remarkably, loss of *Fra-2* in differentiated mKCs (*Fra-2*$^{\Delta sb}$) was sufficient to cause this phenotype. Collectively, these findings show that besides epidermal differentiation defects, loss of epidermal Fra-2 results in skin and systemic inflammation.

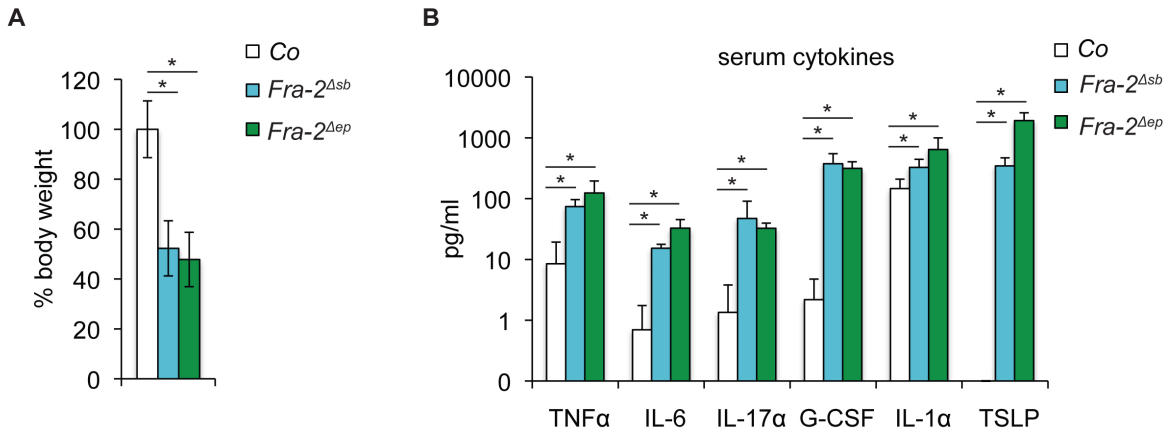


Figure 23. Loss of epidermal Fra-2 results in systemic inflammation. (A) Body weight analysis and **(B)** serum cytokine analysis of *Co*, *Fra-2*$^{\Delta sb}$ and *Fra-2*$^{\Delta ep}$ pups at P6; n=8/genotype; * p<0.05, bars represent mean ±SD.

2.2. Soluble factors associated with inflammation are expressed in epidermal cells upon loss of Fra-2

Next, I addressed the mechanisms by which loss of Fra-2 in epithelial cells causes a systemic phenotype and which keratinocyte-derived factors are involved in this inflammatory response.

Upon loss of Fra-2 in mKCs the expression of soluble, inflammation-associated factors by epidermal cells such as *Lipocalin-2* (*Lcn2)*, the S100 genes *S100A8*, *S100A9* as well as the cytokine *Thymic Stromal Lymphopoietin* (*TSLP)*, a marker of skin barrier defects, was massively increased **(Figure 24)**. Interestingly, it was previously shown that these chemokines and cytokines are causally involved in the development and progression of inflammatory skin conditions (Demehri et al., 2008; Dumortier et al., 2010; Schonthaler et al., 2014). Additionally, it was suggested that these factors are regulated by NF-κB/p65 (Lee and Ziegler, 2007; Nemeth et al., 2009; Zhao and Stephens, 2013).

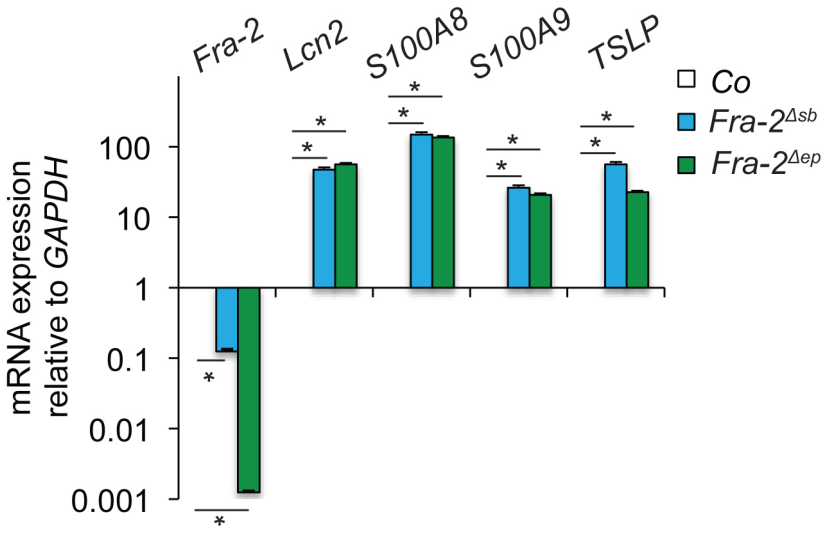


Figure 24. Secretion of inflammation-associated factors by epidermal cells upon loss of Fra-2. Differential *Fra-2*, *Lipocalin 2* (*Lcn2*), *S100A8*, *S100A9* and *Thymic Stromal Lymphopoietin (TSLP)* gene expression in *Fra-2*$^{\Delta sb}$ and *Fra-2*$^{\Delta ep}$ mutants compared to control littermates at P6; n=6/genotype; * p<0.05, bars represent mean ±SD.

2.3. Cell autonomous activation of p65 in Fra-2-deficient mKCs

Since increased expression of p65 targets was detected upon loss of Fra-2 in epidermal keratinocytes, the activity of this TF was evaluated. Notably, increased p65 TF activity was measured in epidermal nuclear lysates upon loss of Fra-2 using a DNA-binding ELISA kit **(Figure 25A)**. This finding was confirmed in primary *Fra-2*$^{f/f}$ mKCs. Upon *AdCre*-mediated *ex vivo* deletion of *Fra-2*, increased p65 TF activity was detected compared to *AdGFP* infected primary mKCs **(Figure 25B)**.

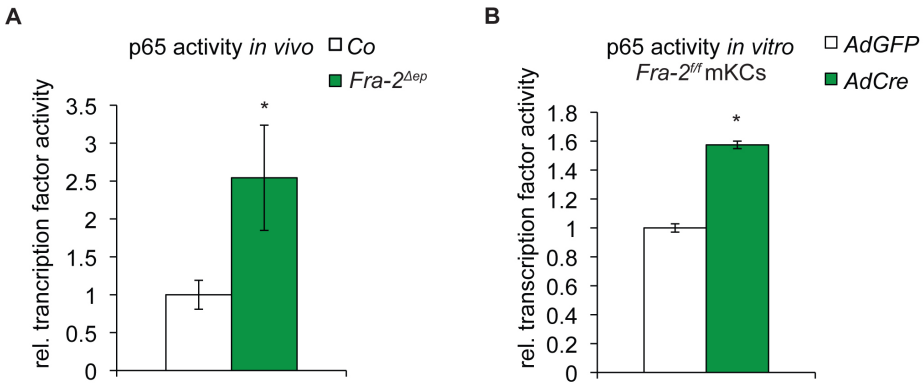


Figure 25. Increased p65 activity in the absence of Fra-2. p65 transcription factor activity measured in epidermal lysates obtained from *Co* and *Fra-2*$^{\Delta ep}$ pups at P6; n=6/genotype **(A)** and primary *Fra-2*$^{f/f}$ mKCs infected with *AdGFP* and *AdCre in vitro*; n=4/genotype **(B)**; * p<0.05, bars represent mean ±SD.

2.4. Skin phenotype and inflammation are ameliorated in *Fra-2/p65$^{\Delta ep}$ DKO* mutants

To evaluate the contribution of increased p65 activity in epidermal cells of *Fra-2$^{\Delta ep}$* mutants, *Fra-2/p65$^{\Delta ep}$* double-knock-out (*DKO*) mutants were generated. Efficient deletion of epidermal Fra-2 and p65 was confirmed by IHC **(Figure 26A,B)**.

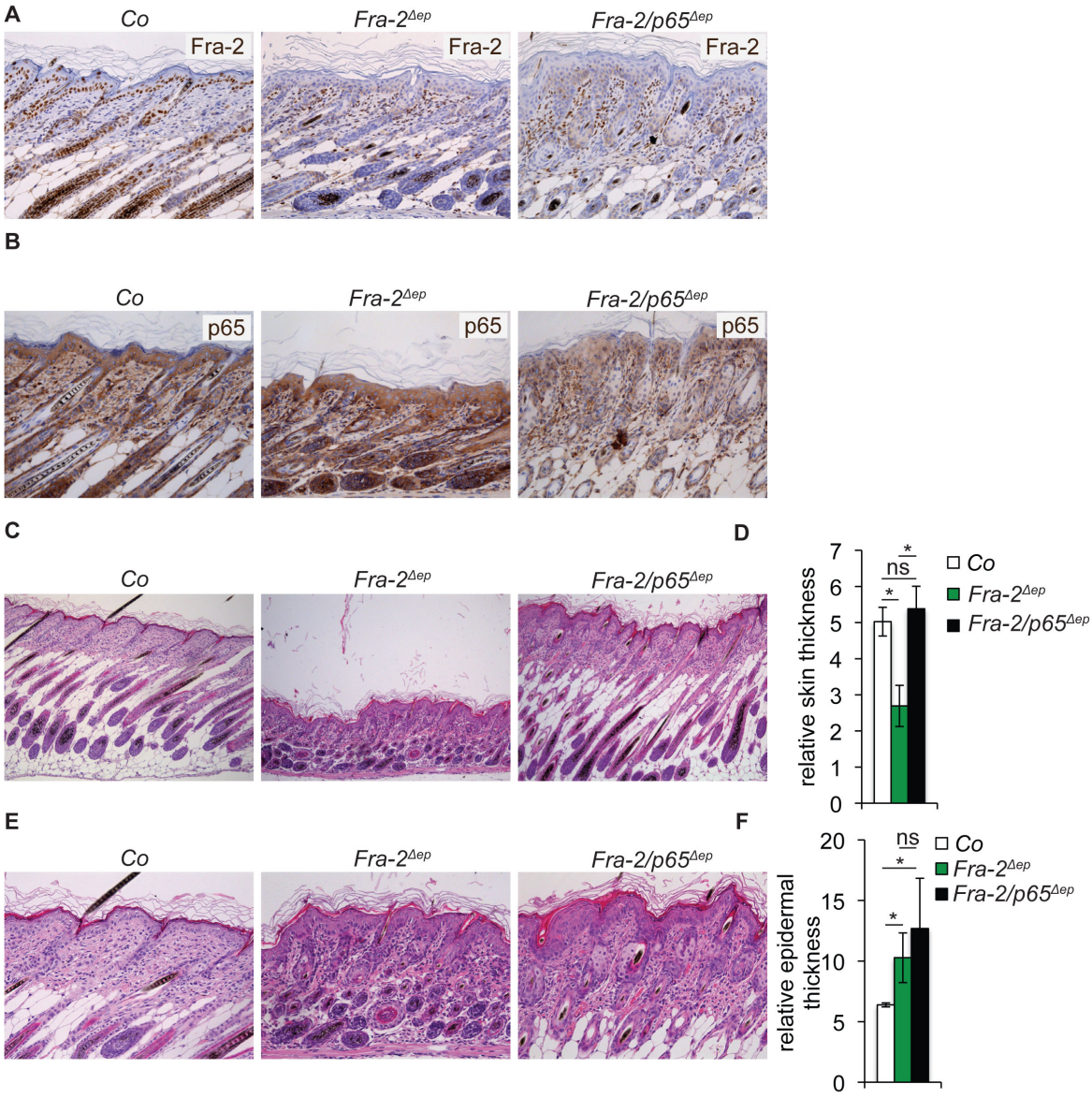


Figure 26. Loss of epidermal p65 ameliorates the skin phenotype of *Fra-2$^{\Delta ep}$* mutants. (A) Fra-2 IHC, **(B)** p65 IHC and H&E analyses to evaluate total skin thickness **(C,D)** and epidermal thickness **(E,F)** of *Co*, *Fra-2$^{\Delta ep}$* and *Fra-2/p65$^{\Delta ep}$ DKO* pups at postnatal day (P6); n=8/genotype; * p<0.05, bars represent mean ±SD.

Remarkably, the skin phenotype was ameliorated in *DKO* mutants. The thickness of the dermal layer was recovered in *Fra-2/p65$^{\Delta ep}$* mutants and hair follicle formation was

comparable to control littermates **(Figure 26C; quantified in Figure 26D)**. However, as observed in *Fra-2*$^{\Delta ep}$ mutants, *Fra-2/p65*$^{\Delta ep}$ *DKO* mutants still presented epidermal hyperplasia with increased epidermal thickness **(Figure 26E; quantified in Figure 26F)**. IHC analyses showed that the epidermal hyperplasia of DKO mutants was marked by keratinocyte hyperproliferation, depicted by increased numbers of Ki67 positive keratinocytes **(Figure 27A; quantified in Figure 27B)**, and increased numbers of CD45 positive inflammatory infiltrates predominantly in the upper dermis, comparable to *Fra-2*$^{\Delta ep}$ mutants **(Figure 27C; quantified in Figure 27D)**.

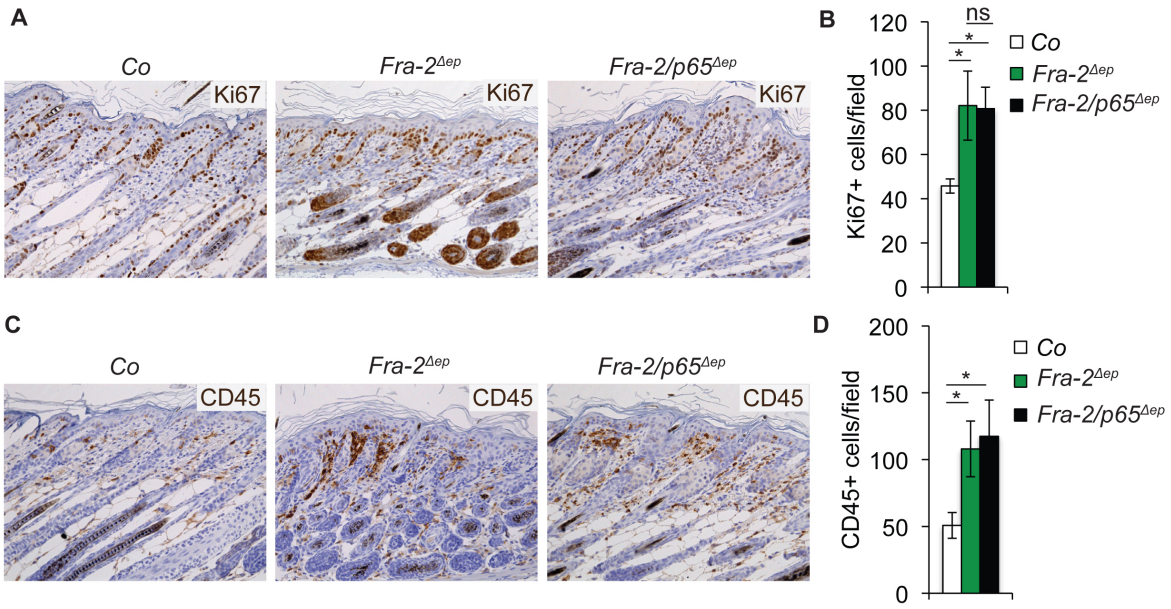


Figure 27. Keratinocyte hyperproliferation and skin inflammation is not rescued in *Fra-2/p65*$^{\Delta ep}$ *DKO* mutants. (A) Ki67 IHC, **(B)** quantification of Ki67 positive cells, **(C)** CD45 IHC, **(D)** quantification of CD45 positive cells of *Co*, *Fra-2*$^{\Delta ep}$ and *Fra-2/p65*$^{\Delta ep}$ *DKO* pups at P6; n=6/genotype; * p<0.05, bars represent mean ±SD.

Even though the skin phenotype of *Fra-2/p65*$^{\Delta ep}$ *DKO* mutants was only partially improved, the systemic response was greatly improved with a significant increase in body weight from 50 to 80% of control littermates **(Figure 28A)**. Additionally, the serum levels of the cytokines IL-6, IL-17A and G-CSF were restored to the levels of littermate controls, the levels of TNFα were partially restored, while the levels of IL-1α and TSLP remained significantly elevated **(Figure 28B)**.

In summary, these genetic experiments demonstrate that skin and systemic inflammation in *Fra-2*$^{\Delta ep}$ mutants are partially regulated by NF-κB/p65 activation in mKCs. Even though the epidermal hyperplasia was not restored in *Fra-2/p65*$^{\Delta ep}$ mutants, the amelioration of

the systemic inflammation marked by reduced levels of several serum cytokines suggested that p65 in mKCs co-regulates the expression of soluble factors.

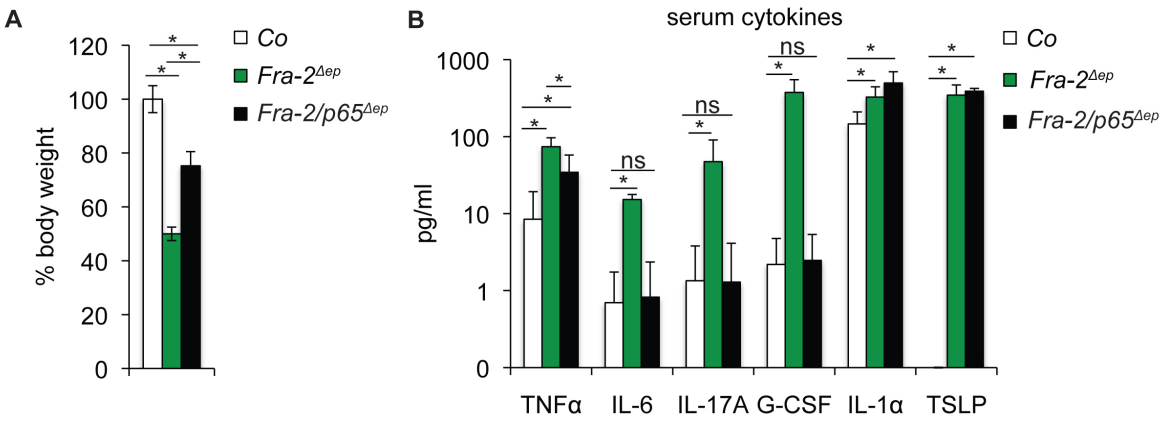


Figure 28. Systemic inflammation ameliorated in *Fra-2/p65$^{\Delta ep}$ DKO* mutants. (A) Body weight analysis and **(B)** serum cytokine analysis of *Co*, *Fra-2$^{\Delta ep}$* and *Fra-2/p65$^{\Delta ep}$ DKO* pups at P6; n=8/genotype; * p<0.05, bars represent mean ±SD.

2.5. Expression of NF-κB-dependent and independent soluble factors by epidermal cells upon loss of Fra-2

Since the mentioned soluble factors expressed upon loss of epidermal Fra-2 have been linked to NF-κB signaling, their expression was evaluated in epidermal samples of *Fra-2/p65$^{\Delta ep}$ DKO* mutants. Notably, the expression of *Lcn2, S100A8* and *S100A9* was significantly reduced compared to *Fra-2$^{\Delta ep}$* mutants but still increased compared to control littermates. However, *TSLP* expression was not reduced compared to *Fra-2$^{\Delta ep}$* mutants **(Figure 29A)**. These findings show that increased p65 activity contributes to the increased expression of *Lcn2*, *S100A8* and *S100A9* upon loss of Fra-2. This might explain the amelioration of the systemic inflammatory response observed in *Fra-2/p65$^{\Delta ep}$ DKO* mutants. Surprisingly and in contrast to what has been suggested in published reports (Lee et al., 2008; Lee and Ziegler, 2007), the expression of TSLP by epidermal cells was NF-κB/p65-independent, which was confirmed at the protein level by ELISA **(Figure 29B)**. These experiments suggest an important function of p65 in Fra-2-deficient mKCs. Inflammation-associated factors expressed by keratinocytes might activate different subsets of immune cells to secrete additional inflammatory mediators. Collectively, reduced expression of some chemokines and cytokines by epidermal cells of *Fra-2/p65$^{\Delta ep}$ DKO* mutants might result in reduced serum cytokines produced by immune cells and hence reduced systemic inflammation.

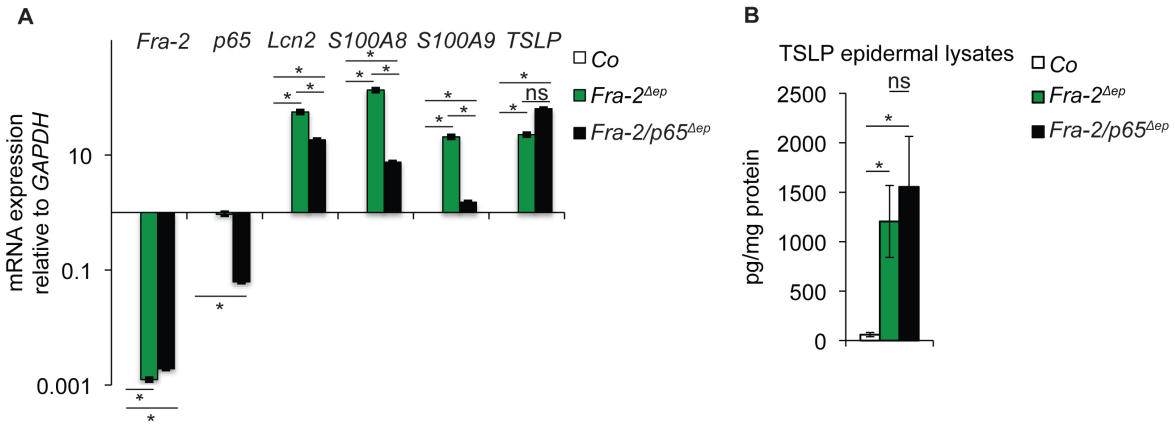
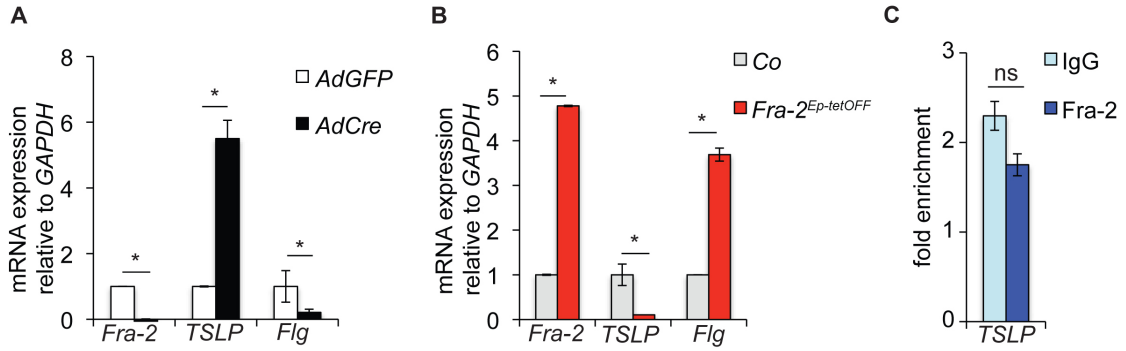


Figure 29. Secretion of inflammation-associated factors partially restored in *Fra-2/p65*$^{\Delta ep}$ *DKO* mutants. (A) Differential *Fra-2, p65, Lcn2, S100A8, S100A9* and *TSLP* gene expression and **(B)** TSLP protein expression in *Fra-2*$^{\Delta ep}$ and *Fra-2/p65*$^{\Delta ep}$ *DKO* mutants compared to control littermates at P6; n=6/genotype; * p<0.05, bars represent mean ±SD.

2.6. Fra-2 indirectly regulates epithelial-derived TSLP expression

TSLP was consistently up-regulated in *Fra-2*$^{\Delta ep}$ mutants and found independently regulated by NF-κB/p65. We next evaluated *TSLP* expression upon loss of/increased *Fra-2* expression in primary mKCs *in vitro* to assess its cell-autonomous regulation by Fra-2. As shown previously, *Fra-2* expression correlated with the expression of the terminal differentiation gene *Flg*. Surprisingly, an inverse correlation of *Fra-2* and *TSLP* expression in primary mKCs was found: *TSLP* expression by mKCs upon loss of *Fra-2* was increased while it was blocked upon increased *Fra-2* expression **(Figure 30A,B)**. Interestingly, ChIP experiments failed to detect binding of Fra-2 to an AP-1 consensus site in the *TSLP* promoter **(Figure 30C)**. These findings indicate that even though the expression of *TSLP* is modulated cell autonomously by Fra-2, it is likely not regulated by direct binding of Fra-2 to the *TSLP* promoter. In summary, these experiments suggest that the expression of TSLP is inversely correlated with the differentiation status of primary mKCs.



Figrue 30. TSLP expression is indirectly regulated by Fra-2 in primary mKCs. (A,B) Differential *Fra-2*, *TSLP* and *Flg* expression in primary Fra-2-deficient **(A)** and *Fra-2*$^{Ep\text{-}tetOFF}$ mKCs **(B)** compared to control cells. **(C)** ChIP analysis of Fra-2 binding to an AP-1 consensus site in the TSLP promoter; n=4; * p<0.05, bars represent mean ±SD.

2.7. Transplantation of *Fra-2*$^{\Delta ep}$ skin to immune-deficient mice results in the formation of skin tumors

As *Fra-2*$^{\Delta ep}$ mutants died within 2 weeks after birth, skin transplantation assays were performed to monitor the progression of the inflammatory skin phenotype. Skin of control as well as *Fra-2*$^{\Delta ep}$ pups at P2 was grafted onto immune-compromised SCID (severe combined immunodeficiency) mice and analyzed after 4 weeks. Interestingly, Fra-2-deficient skin grafts gave rise to skin papillomas in the absence of functional B and T cells **(Figure 31A)**. The lesions of *Fra-2*$^{\Delta ep}$ grafts presented epidermal hyperplasia, hyper- and parakeratosis and atypia 4 weeks after transplantation **(Figure 31B)**. The transplanted skin of *Fra-2*$^{\Delta ep}$ mutants was highly proliferative **(Figure 31C)** and presented massive infiltration of CD45 positive immune cells **(Figure 31D)**. We hypothesize that the absence of T cells might be the cause of skin papilloma formation in *Fra-2*$^{\Delta ep}$ grafts since it has been demonstrated that T cells, activated by TSLP, cause a tumor protective microenvironment (Demehri et al., 2012; Di Piazza et al., 2012). These findings are of high relevance because the blockade of TSLP has been suggested to be a promising therapeutic strategy to treat AD patients.

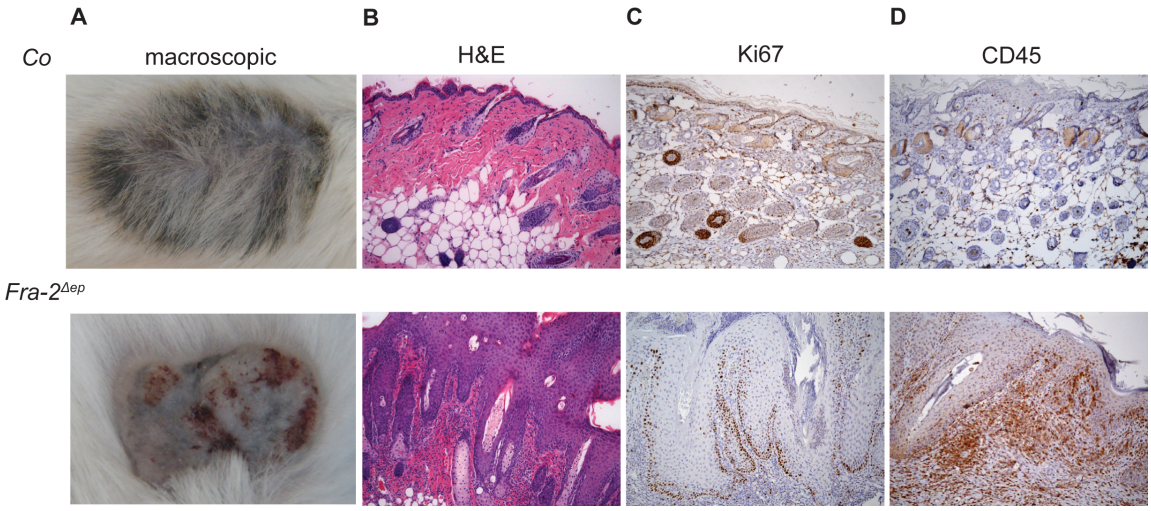


Figure 31. *Fra-2*$^{\Delta ep}$ skin grafts give rise to papillomas. (A) Macroscopic appearance, (B) microscopic appearance, (C) Ki67 IHC and (D) CD45 IHC of *Co* and *Fra-2*$^{\Delta ep}$ skin grafts 4 weeks after transplantation; n=6/genotype.

Discussion

In this thesis I describe how the AP-1 transcription factor Fra-2 controls epidermal barrier homeostasis by a dual mechanism: First, I demonstrate that Fra-2 regulates the expression of epidermal differentiation genes upon interaction with Ezh2 and ERK1/2. Second, I show that this mechanism is essential and that the absence of Fra-2 in epidermal keratinocytes causes an inflammatory skin phenotype characterized by the secretion of cytokines and chemokines by keratinocytes and the activation of NF-κB.

1.1. Functions of Fra-2 during keratinocyte differentiation

In the first part of this study, I have identified Fra-2 as a novel Ezh2 substrate in basal cells of the epidermis, which acts as a key regulator of terminal epidermal differentiation. Mechanistically, Fra-2 binds conserved AP-1 consensus sites in EDC promoters and induces transcriptional activation, when phosphorylated by ERK1/2. Unlike other master regulators of terminal epidermal differentiation such as Klf4, which is expressed suprabasally (Segre et al., 1999), Fra-2 is expressed and bound at EDC promoters in basal and differentiated keratinocytes. In basal cells, Fra-2 is unphosphorylated, methylated on K104 and transcriptionally inactive. Using genetic loss of function approaches in mice, we demonstrate that loss of Fra-2 in suprabasal cells is sufficient to cause skin barrier defects. These data support the observation that even though expressed in all epidermal keratinocytes, Fra-2 is only active and mainly functions in the differentiated layers of the epidermis. Conversely, we show that ectopic Fra-2 expression is sufficient to induce terminal epidermal differentiation leading to suppression of papilloma growth by inducing precocious keratinocyte differentiation.

1.2. Fra-2 is kept transcriptionally inactive by methylation

Given our *in vitro* and *in vivo* data, I speculate that Fra-2 is kept transcriptionally inactive in basal cells due to methylation by Ezh2. Upon Ca^{2+}-induced differentiation, the concomitant loss of interaction with Ezh2, loss of Fra-2 methylation and ERK1/2-mediated phosphorylation correlates with EDC gene expression and keratinocyte differentiation. Using Ca^{2+} treatment to induce keratinocyte differentiation, I demonstrate that basal keratinocytes differentiate prematurely upon inhibition of Ezh2. These findings are in agreement with published results of *in vivo* gene targeting of Ezh2 in the epidermis. Ezh2-null keratinocytes differentiate prematurely and express higher levels of EDC genes with increased AP-1 transcription factor activity (Ezhkova et al., 2009). It was previously shown

that Ezh2 can modify the activity of some transcription factors through methylation (He et al., 2012; Kim et al., 2013; Lee et al., 2012; Xu et al., 2012). Employing MS-based approaches, we identified mono- and dimethylation on K104 of Fra-2 in basal mKCs. To our knowledge, this is the first work showing methylation of an AP-1 transcription factor. Additionally, I demonstrate by lentiviral expression of a Fra-2 mutant mimicking lysine methylation that this modification decreases the transcriptional activity of Fra-2 on EDC genes. Interestingly, K104 of Fra-2 was found acetylated in an independent study (Park et al., 2013), suggesting a potential role of this residue in regulating the transcriptional activity of this protein. We hypothesize that Fra-2 K104 methylation influences the interaction of Fra-2 with transcriptional co-activators, as it has been described for methylated GATA4 (He et al., 2012).

During the last decade several independent studies established that Ezh2 is overexpressed in solid tumors, and is associated with aggressive cancers and progression (Hock, 2012). In human cancers, whether H3K27 methylation is the only way by which Ezh2 controls tumorigenesis remains unclear. Since Ezh2 has other substrates beyond histone H3, it was proposed that Ezh2 might facilitate tumorigenesis by regulating the activity of non-histone substrates such as transcription factors (He et al., 2012; Kim et al., 2013). Interestingly, skin papillomas of poorly differentiated *K5-SOS-F* mice show high levels of Ezh2 and express Fra-2. Importantly, ectopic expression of Fra-2 in this background resulted in reduced papilloma growth due to precocious differentiation. These findings suggest that the ratio of expression of Ezh2 to its substrates, among them Fra-2, is crucial in regulating epidermal differentiation and tumorigenesis. Further research will be necessary to assess the expression of Ezh2 and Fra-2 in human skin diseases characterized by epidermal differentiation defects, in order to evaluate the possible clinical benefit of blocking Ezh2 as a therapeutic strategy.

1.3. Increased transcriptional activity of Fra-2 upon C-terminal phosphorylation

Additionally, we identify an important function of the MAP kinases ERK1/2 in terminal mKC differentiation by phosphorylating and stabilizing Fra-2. ERK1/2 are activated upon Ca^{2+}-induced differentiation via PKCα and the inhibition of ERK1/2 results in reduced expression of terminal keratinocyte differentiation genes (Schmidt et al., 2000; Seo, 2004). I provide further mechanistic insights demonstrating that Fra-2 is phosphorylated by ERK1/2 and acts as an ERK1/2-dependent inducer of terminal differentiation by regulating

EDC gene expression. Additionally, *in vivo* analyses of ERK1/2 phosphorylation in developing embryos showed a clear nuclear pattern in suprabasal cells indicating essential functions of activated ERK1/2 in epidermal barrier acquisition. Previously published studies revealed that ERK2 phosphorylates Fra-2 on 5 serine and threonine residues *in vitro* (Alli et al., 2013). Targeted mutagenesis of these residues demonstrated that the C-terminal phosphorylation of Fra-2 on serine 320 and threonine 322 results in increased protein stability (Alli et al., 2013). Using an antibody against phospho serine 320, which is conserved among Fra-1, Fra-2 and c-Fos, we found that C-terminal Fra-2 phosphorylation is increased upon mKC differentiation correlating with ERK1/2 activation and Fra-2 transcriptional activity at EDC target genes. In addition, I functionally validated the role of C-terminal Fra-2 phosphorylation in primary mKCs by expression of phospho-deficient Fra-2 mutants, which exhibited reduced transcriptional activity.

I propose a novel mechanism how Fra-2/AP-1 activity is regulated through lysine methylation by Ezh2 and C-terminal serine phosphorylation by ERK1/2 in mKCs. It will be interesting to explore in future studies whether this methyl-phospho switch is a general mechanism of transcription factor regulation and to study the possible implications in skin diseases and beyond.

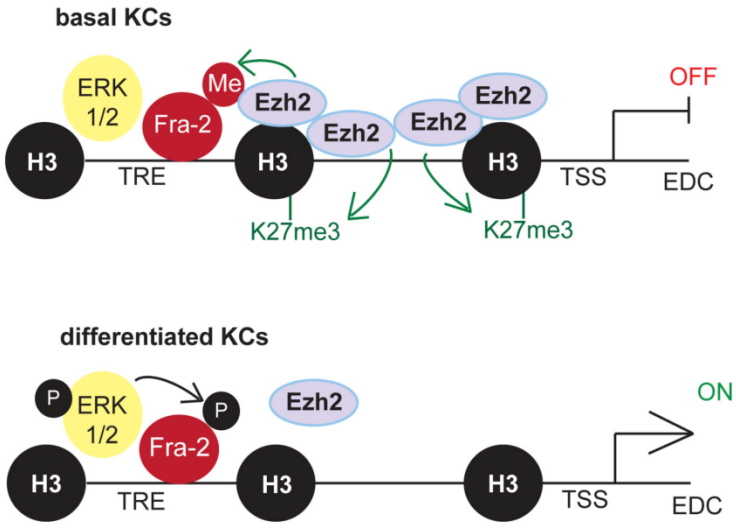


Figure 1-D. Proposed model of transcriptional regulation of EDC genes by Fra-2, Ezh2 and ERK1/2.

1.4. Fra-2 expression in suprabasal cells is necessary for skin barrier acquisition

Using LOF and GOF approaches in mouse models and primary keratinocyte cultures, I have identified Fra-2 as a key activator of terminal keratinocyte differentiation. The finding

that loss of Fra-2 specifically in differentiated keratinocytes gives rise to epidermal barrier defects, similar to those observed when Fra-2 is deleted in both basal and differentiated keratinocytes, supports the conclusion that epidermal Fra-2 is an essential regulator of terminal keratinocyte differentiation. Reduced expression of epidermal differentiation genes such as *Flg* upon Fra-2 loss is likely responsible for the barrier defect. Using a mouse model that lacks processed murine Flg due to a frameshift mutation in the gene encoding *profilaggrin*, which mimics mutations found in 9% of human AD cases, it was demonstrated that Flg deficiency alone decreases stratum corneum hydration and leads to skin barrier abnormalities (Scharschmidt et al., 2009). However, in around two thirds of AD patients with skin barrier deficiencies, Flg is properly expressed (Jakasa et al., 2011). This points to the existence of different subtypes of AD in humans that might be caused due to reduced expression of other epidermal differentiation genes, or deregulated expression of upstream regulators such as transcription factors.

2.1. Loss of epidermal Fra-2 – a mouse model to study inflammatory skin diseases

In human skin diseases, a defect in the tightly orchestrated, stepwise process of epidermal differentiation has been associated with epidermal hyperplasia and skin inflammation. Previous studies have established that keratinocyte hyperproliferation is a compensatory response and secondary to epidermal barrier defects (Matsuki et al., 1998; Proksch et al., 1991; Segre et al., 1999) and that the activation of inflammation-associated signaling pathways, such as NF-κB are crucially involved in the initiation of skin inflammation. Besides epidermal barrier defects, mutant mice with epithelial-specific deletion of Fra-2 exhibit skin lesions with clinical features reminiscent to the ones observed in patients with AD or psoriasis. First, Fra-2 LOF mutants exhibit epidermal hyperplasia characterized by increased epidermal proliferation, as observed in AD and psoriasis patients (Proksch et al., 2008; Wagner et al., 2010). Second, Fra-2-deficient keratinocytes show dramatically increased expression of the Th2-type cytokine *TSLP*, which is crucially involved in the initiation of AD (Ziegler and Artis, 2010) as well as increased expression of the chemokines *S100A8*, *S100A9* and *Lcn2*, which have been linked to the pathogenesis of psoriasis (Kamata et al., 2012; Schonthaler et al., 2014). Third, activation of NF-κB was found in these mutant mice. The activation and nuclear translocation of NF-κB is often associated with increased epidermal proliferation and is considered a critical event in the

progression and maintenance of AD as well as psoriasis (Dajee et al., 2006; Lizzul et al., 2005). Importantly, loss of suprabasal Fra-2 is sufficient to cause these phenotypic features. Taking these findings together, I propose that mouse models with epidermal Fra-2 deletion may serve as useful tools to study the pathology of inflammatory skin diseases such as AD and psoriasis.

2.2. Expression of soluble factors in mKCs upon loss of epidermal Fra-2

Epidermal keratinocytes are key initiators of immune responses through the secretion of soluble factors that induce immune cell activation and thereby adaptive immune responses (Holgate, 2007). In this study, increased expression of the NF-κB/p65 targets *S100A8/A9*, *Lcn2* and *TSLP* by epidermal cells is found upon loss of Fra-2. Previous studies established that the expression of these soluble factors is linked to skin barrier defects and that increased expression of *TSLP* is sufficient to initiate skin inflammation (Demehri et al., 2008; Kamata et al., 2012; Yoo et al., 2005; Zenz et al., 2005). Additionally, increased p65 activity is detected in Fra-2-deficient keratinocytes. Employing *Fra-2/p65$^{\Delta ep}$ DKO* mutant mice, we genetically demonstrate that the absence of p65 results in reduced expression of *S100A8/A9* and *Lcn2* and thereby validate that p65 contributes to regulate their expression. Since *S100A8/A9* and *Lcn2* affect the activity of various immune cells, reduced expression of these soluble factors by mKCs might explain the amelioration of the systemic inflammatory response, assessed by reduced levels of the inflammatory/hematopoietic cytokines IL-6, IL-17 and G-CSF in sera of *Fra-2/p65$^{\Delta ep}$ DKO* mutants. Reduced levels of these cytokines may also explain the improved body weight of *Fra-2/p65$^{\Delta ep}$ DKO* mutant mice compared to *Fra-2$^{\Delta ep}$* mutant mice. However, high serum levels of IL-1α, TNFα and TSLP were still measured also in the absence of p65 in *Fra-2/p65$^{\Delta ep}$ DKO* mutants. These results demonstrate that upon loss of Fra-2, p65/NF-κB in keratinocytes is involved in initiating inflammation, but that loss of Fra-2 affects the expression of specific cytokines in a p65-independent manner.

2.3. Epidermal p65 does not regulate epidermal hyperplasia and skin inflammation upon skin barrier defects

The use of mice lacking IκBα, the main inhibitory protein that controls NF-κB activation, has shown that increased NF-κB activation triggers increased keratinocyte proliferation, epidermal hyperplasia and immune cell infiltration in the dermis and in the epidermis

(Klement et al., 1996). While the differentiation status of epidermal keratinocytes remained unaffected upon loss of p65, epidermal p65 was found to be essential for DMBA-/TPA-induced skin carcinogenesis. Moreover, keratinocyte-specific p65 deficiency prevents TPA-induced epidermal hyperproliferaiton and the expression of inflammatory cytokines and chemokines by keratinocytes (Kim and Pasparakis, 2014). Even though *Fra-2/p65$^{\Delta ep}$ DKO* mice show striking microscopic improvements in skin architecture, as judged by total skin thickness and hair follicle formation, epidermal hyperplasia is not rescued. These findings suggest that keratinocyte hyperproliferation in this model is not regulated by epidermal p65, but likely triggered by epidermal differentiation defects,.

Notably, as observed in *Fra-2$^{\Delta ep}$* mutants, the skin of *Fra-2/p65$^{\Delta ep}$ DKO* mutants exhibits massive infiltration of CD45 positive immune cells, indicating that p65 activity is not necessary for the recruitment of inflammatory cells. Since this finding is contradictory to the result that *Fra-2/p65$^{\Delta ep}$ DKO* mutant mice show reduced levels of several inflammatory/hematopoietic cytokines in the serum, it would be interesting to analyze in detail the subtypes of immune cells present in *Fra-2$^{\Delta ep}$* and *Fra-2/p65$^{\Delta ep}$ DKO* skin. Moreover, I suggest measuring the secretion of cytokines to evaluate whether different populations are involved in the skin inflammatory response in *Fra-2/p65$^{\Delta ep}$ DKO* mice. The findings that epidermal hyperplasia and skin inflammation are still present upon removal of p65 suggest that these features might be secondary to epidermal differentiation defects upon loss of Fra-2 and are not directly linked to p65 activity. For all these reasons, the inhibition of p65 might not be a valuable therapeutic strategy for inflammatory skin diseases characterized by altered epidermal differentiation. The deletion of p65 in mKCs of *Fra-2$^{\Delta ep}$* mutants, a mouse model of skin inflammation characterized by skin barrier defects, only partially ameliorates the systemic inflammatory response, likely due to reduced expression of keratinocyte-derived chemokines, while epidermal hyperplasia and skin inflammation remain unaffected.

2.4. Indirect regulation of TSLP by Fra-2

The expression of the cytokine TSLP by keratinocytes was initially suggested to be a consequence of skin barrier defects as observed in a mouse model upon loss of Notch signaling in the developing epidermis (Demehri et al., 2008) and was linked to NF-κB pathway activation (Briot et al., 2009; Dumortier et al., 2010). TSLP, a Th2 cytokine, has been studied intensively in the pathogenesis of allergic inflammation and subsequent

development of AD and other immune disorders (Liu, 2006; Soumelis et al., 2002; Yoo et al., 2005). The role of TSLP in AD skin lesions is to promote the recruitment, proliferation and maturation of Th2 immune cells to the lesional site. Here I show that TSLP expression is regulated cell autonomously upon loss or increased expression of Fra-2 in keratinocytes. Fra-2 ChIP experiments and the analyses of *Fra-2/p65$^{\Delta ep}$ DKO* mice seem to exclude a direct transcriptional regulation of Fra-2 and p65 on the TSLP promoter, even though several reports suggest a transcriptional function of AP-1 and NF-κB TFs on the transcriptional regulation of TSLP (Dumortier et al., 2010; Lee et al., 2008; Redhu et al., 2011). More extensive analyses, in particular binding of other members of the AP-1 and NF-κB TF family to the TSLP promoter will be required to understand the transcriptional regulation of this gene.

2.5. Expression of TSLP inversely correlated with the differentiation status of mKCs

Even though our studies highlight a cell autonomous regulation of TSLP expression that is inversely correlated with the expression of Fra-2 and the differentiation status of mKCs, the transcriptional regulation of TSLP upon keratinocyte differentiation defects remains largely unknown.

Interestingly, one recent study linked TSLP expression to an increase in intracellular Ca^{2+} levels and the activation of NFAT transcription factors (Wilson et al., 2013). Given the fact that a calcium gradient is required for efficient mKC differentiation *in vitro* and *in vivo* (Menon et al., 1985) and that this calcium gradient is lost in inflammatory skin disorders (Menon et al., 1992), the question arises whether incomplete mKC differentiation results in altered intracellular calcium levels and subsequent changes in the activity of calcium-sensing TFs, such as members of the NFAT family. Hypothetically, if a differentiation defect results in an increase of intracellular calcium levels, this might activate calcium-binding proteins such as the S100 chemoattractants, which are causally involved in the pathogenesis of inflammatory skin diseases. Additionally, the concept of differentiation-regulated calcium levels might explain reduced TSLP expression upon increased expression of EDC genes in mKCs ectopically expressing Fra-2. Therefore, I plan to measure intracellular calcium levels, the activity of NFAT transcription factors and the expression of calcineurin, the phosphatase that activates NFAT TFs (Loh et al., 1996), upon loss and increased expression of Fra-2 in mKCs. If the hypothesis holds true that the

expression of TSLP is regulated by calcium levels, which depend on the differentiation status of keratinocytes, this cytokine would present an important link how skin barrier defects cause skin inflammation.

2.6. Fra-2 – p65 interaction: competition for co-activators?

The question how p65 is activated in the absence of Fra-2 remains to be answered. Early studies suggest an interaction of AP-1 and NF-κB TFs via the co-activator p300 or its homolog CBP (Gerritsen et al., 1997; Torchia et al., 1998). In these reports, the concept of competition of AP-1 and NF-κB TFs for interaction with p300/CBP was proposed (Sheppard et al., 1998). Interestingly, the acetylation of p65 by the acetyltransferases p300/CBP on lysine 310 has been linked to increased transcriptional activity (Chen et al., 2002). In future studies I plan to evaluate whether the interaction with p300 and the acetylation of p65 on lysine 310 is increased in mKCs upon loss of Fra-2 by the use of coIP experiments, MS and Western blot analyses. The hypothesis is that upon loss of Fra-2, p65 can interact more easily with the transcriptional co-activators and acetyltransferases p300/CBP, increasing its transcriptional activity and thereby inducing the expression of its target genes.

2.7. Linking altered epidermal differentiation, skin inflammation and skin cancer

I have shown in this thesis that the AP-1 transcription factor Fra-2 regulates epidermal barrier immunity at two levels: by transcriptionally activating the expression of epithelial differentiation genes and by keeping inflammatory mediators, such as the TF p65 and inflammation-associated soluble factors, under control.

While most of these results were obtained from studying Fra-2 LOF mouse models, some of these findings were confirmed using an epithelial-specific Fra-2 GOF mouse model. We found that keratinocytes differentiated prematurely and the expression of TSLP was blocked upon exogenous *Fra-2* expression. Additionally, I have shown a functional relevance of ectopic Fra-2 expression *in vivo* in a papilloma-prone mouse model, which presents skin barrier defects. *K5-SOS-F* transgenic mice that ectopically express *Fra-2* show smaller lesions, which are more differentiated. These findings indicate important functions of Fra-2 and the expression of terminal differentiation genes in skin tumors.

Importantly, transplantation of *Fra-2*$^{\Delta ep}$ mutant skin to immune-compromised mice, which lack functional B and T cells, gave rise to the formation of skin papillomas. A recent

concept suggests that TSLP, secreted by keratinocytes upon barrier defects, establishes a tumor protective environment by activating T cells (Demehri et al., 2012; Di Piazza et al., 2012). Along these lines, results from our transplantation model imply that the absence of B and T cells in barrier-defective skin may promote the development of skin papillomas that might progress to invasive SCCs.

Additional studies are necessary to understand the two faces of inflammation and tumor development in skin. In this thesis, I concentrated on the question of how epidermal differentiation defects give rise to skin inflammation. I have shown that the activity of the transcription factor Fra-2 is crucial during epidermal development and it is regulated by Ezh2- and ERK1/2-dependent posttranslational modifications. Incomplete epidermal differentiation results in skin inflammation likely due to cell autonomous activation of NF-κB and the expression of the cytokine TSLP by keratinocytes. Since proper epidermal differentiation and the expression of inflammatory mediators by keratinocytes are crucially involved in the initiation of inflammatory skin diseases and skin tumorigenesis, my work has provided a basis for future work aiming at preventing, detecting or curing skin diseases.

Conclusions

Conclusions

1) Fra-2 functions as a key regulator of keratinocyte differentiation by inducing the expression of epidermal differentiation genes upon interaction with Ezh2 and ERK1/2. Fra-2 activity is regulated by lysine methylation in basal keratinocytes and by C-terminal phosphorylation upon keratinocyte differentiation.

2) Ectopic Fra-2 expression induces epidermal differentiation *in vivo* during development and suppresses skin papilloma growth due to increased expression of epidermal differentiation genes.

3) Fra-2 expression is necessary for proper epidermal differentiation and skin barrier acquisition.

4) Loss of epidermal Fra-2 results in inflammation characterized by epidermal hyperplasia, infiltration of inflammatory cells into the skin, increased activity of p65 in keratinocytes, secretion of inflammation-associated factors by epidermal cells and high serum cytokine levels.

5) Concurrent deletion of Fra-2 and p65 results in an amelioration of the systemic phenotype, likely due to reduced expression of inflammation-associated soluble factors by epidermal cells, but does not rescue keratinocyte hyperproliferation and skin inflammation observed upon loss of epidermal Fra-2.

6) Expression of Fra-2 and EDC genes is inversely correlated with TSLP expression; however, Fra-2 does not appear to regulate TSLP expression by direct promoter binding.

7) Skin barrier defects upon loss of epidermal Fra-2 in an immune-compromised background result in the formation of skin papillomas.

Conclusiones

Conclusiones

1) Fra-2 funciona como un regulador clave de la diferenciación de los queratinocitos mediante la inducción de la expresión de genes de diferenciación epidérmica tras la interacción controlada con Ezh2 y ERK1/2. La actividad de Fra-2 está regulada por la metilación de lisinas en los queratinocitos basales y su fosforilación en posición C-terminal tras la diferenciación.

2) La expresión ectópica de Fra-2 induce la diferenciación epidérmica *in vivo* durante el desarrollo y suprime el crecimiento de los papilomas de la piel debido a un aumento de expresión de genes de diferenciación epidérmica.

3) La expresión de Fra-2 es necesaria para la adecuada diferenciación epidérmica y la adquisición de la función barrera de la piel.

4) La pérdida de la expresión de Fra-2 en la epidermis resulta en una inflamación caracterizada por hiperplasia epidérmica, infiltración de células inflamatorias en la piel, aumento de la actividad de p65 en los queratinocitos, la secreción de factores asociados con la inflamación por las células epidérmicas y niveles de citoquinas séricas elevadas.

5) La eliminación simultánea de Fra-2 y p65 resulta en una mejora del fenotipo sistémico, probablemente debido a la reducción de la expresión de factores solubles asociados a la inflamación por las células epidérmicas, pero no anula la hiperproliferación de queratinocitos y la inflamación de la piel producida tras la pérdida de Fra- 2 en la epidermis.

6) La expresión de Fra-2 y genes EDC está inversamente relacionada con la expresión de TSLP, sin embargo, Fra-2 no regula la expresión de TSLP directamente mediante la unión a su promotor.

7) Los defectos de la función barrera de la piel producidos por la pérdida de Fra-2 en la epidermis, en un modelo inmunodeficiente, dan lugar a la formación de papilomas en la piel.

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Appendix